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Original Article

Rintatolimod: a potential treatment in patients with pancreatic cancer expressing Toll-like receptor 3

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Abstract: Pancreatic cancer has a dismal prognosis, and treatment options for patients with locally advanced or metastatic disease are limited. Early tumor progression after standard chemo- and or radiotherapy remains a major concern in managing these patients. Treating pancreatic cancer patients with the Toll-like receptor 3 (TLR-3) agonist rintatolimod (Ampligen®) was effective in boosting the immune response. Rintatolimod acts via the TLR-3 receptor on several immune cells. However, the TLR-3 expression pattern in pancreatic cancer cells and how rintatolimod affects pancreatic cancer cells have not yet been investigated. The TLR-3 protein and mRNA expression were evaluated in thirteen PDAC tissue samples as well as in the human PDAC (hPDAC) cell lines CFPAC-1, MIAPaCa-2, and PANC-1 using immunohistochemistry and multiplexed gene expression analysis, respectively. The direct anti-tumor effects of rintatolimod were investigated using a proliferation and migration assay after different incubation time points with increasing concentrations of rintatolimod (ranging from 0.05 to 0.4 mg/ml). The TLR-3 protein and mRNA expression were heterogeneous between the PDAC tissue samples and the three hPDAC cell lines. TLR-3 protein and mRNA expression were high in CFPAC-1, moderate in MIAPaCa-2, and undetectable in PANC-1. Rintatolimod three-day treatment resulted in significantly reduced proliferation of CFPAC-1 cells compared to vehicle-treated control cells. In addition, after 24 hours, rintatolimod-treated CFPAC-1 cells showed less cell migration compared to vehicle-treated control cells, although this difference was not statistically significant. Lastly, we identified fifteen genes, altered with a Log_2 FOC > |1.0| in rintatolimod-treated CFPAC-1 cells, which were significantly related to three transcription factors (NFKB1, RELA, and SP1) regulating the TLR-3 signaling pathway. In conclusion, we propose that rintatolimod treatment might have a direct TLR-3-dependent anti-tumoral effect on pancreatic cancer cells expressing TLR-3.

Keywords: PDAC, TLR-3 agonist, direct anti-tumoral effects, cancer cell proliferation, cancer cell migration, rintatolimod (Ampligen®)

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with a five-year overall survival rate of only 6-9%, despite standard therapy with surgery, chemo(radio)therapy, or combination treatments [1, 2]. The management of PDAC is challenged by delayed diagnosis, (epi) genetic heterogeneity, stromal desmoplasia, and a tolerogenic tumor microenvironment [2-5]. In the last decade, research has focused on novel immunotherapeutic agents such as biological immunomodulators targeting Toll-like receptor 3 (TLR-3) to improve survival. TLR-3 is a pattern recognition receptor that senses double-stranded RNA (dsRNA) from the endosomal membrane and is expressed in immune cells and fibroblasts. Interestingly, various studies reported intra- and extracellular TLR-3 protein expression on pancreatic cancer cells [6-10]. In immune cells, TLR-3 activation induces the production of pro-inflammatory cytokines, including type I interferon (IFN-I), essential for innate and adaptive immune res-

ponses. Recent studies reported that TLR-3 activation also exerted direct anti-tumoral effects in cancer cells via induced transcription factor IFN regulatory factor 3 (IRF-3), leading to downstream signaling axes involved in cell cycle regulation [11]. A limited number of clinical trials investigated synthetic TLR-3 agonists (i.e., PolyA.PolyU and PolyI:C12U (rintatolimod)) in patients with solid tumors and showed improved survival rates in patients with breast cancer [12-14]. Corresponding results were found in our data collected according to the immune monitoring protocol (MEC-2016-575) from patients with stable advanced pancreatic cancer stages treated with rintatolimod after initial FOLFIRINOX chemotherapy [15]. However, it remains uncertain whether the improved survival is due to the immune response activation, the potential direct anti-tumoral effect of rintatolimod, or both. Therefore, in this study, we evaluated the TLR-3 protein and mRNA expression in resected PDAC tissue samples and three human pancreatic ductal adenocarcinomas (hPDAC) cell lines. In addition, we investigated the anti-tumoral effect of the TLR-3 agonist rintatolimod in the three hPDAC cell lines in the absence of immune cells, using cell proliferation and migration assays as well as multiplexed gene expression analysis.

Materials and methods

Cell lines and culture conditions

The human pancreatic cell lines PANC-1, MIAPaCa-2, and CFPAC-1 were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were allelotyped, and the short tandem repeat profile corresponded with the profile provided by the ATCC. The cells were cultured in a 75 cm^2 culture flask stored in a humidified incubator at 37°C with an atmosphere of 5% CO_2 and 95% air. The culture medium consisted of RPMI-1640 medium supplemented with 5% Foetal Bovine Serum (FBS), penicillin (1×10^5 U/L), Fungizone (0.5 mg/L), and L-glutamine (2 mmol/L) and cells were periodically confirmed as mycoplasma-free. Cells were harvested with trypsin (0.05%)/EDTA (0.53 mM) solution. Before plating, cells were automatically counted using the Invitrogen Countess Automated Cell Counter™ to determine the desired starting cell densities. Cell viability was determined with Trypan blue staining.

Drugs and reagents

Rintatolimod liquid solution containing 200 mg per 80 ml was obtained from AIM Immunotech, Inc. (Ocala, F.L., USA). Rintatolimod was stored at 2-8°C and constituted according to the manufacturer's instructions. Media and supplements, Dulbecco's Phosphate Buffered Saline (DPBS), and Trypan Blue Stain (0.4%) were obtained from Life Technologies Limited (Paisley, UK). Hoechst 33258 Pentahydrate (bis-Benzimide) stain was purchased from Thermo Fisher Scientific (Waltham, M.A., USA).

Cell proliferation assay

An optimization experiment was performed for every cell line to determine the optimal cell plating density, ensuring that the plated cells were in their logarithmic growth phase during the entire experiment. The optimal plating densities for PANC-1, MIAPaCa-2, and CFPAC-1 were 25,000, 20,000, and 10,000 cells per well. After trypsinization, cells were plated in 1 mL of medium using 24-wells plates. The plates were placed in a 37°C, 5% CO_2 incubator, and cells were allowed to adapt and attach for three days. Thereafter, after washing the plates multiple times with 1 ml of DPBS and 1 ml of medium, the culture medium in each well was replaced with 1 ml of medium containing 0.5% FCS. The cells were treated with four rintatolimod concentrations; 0.05, 0.1, 0.2, and 0.4 mg/ml. In addition, cells were treated with identical concentrations of rintatolimod's vehicle solution ($CaCl_2$ & $MgCl_2$) to rule out any (anti-) proliferative effects of the vehicle solutions. Cells were treated for 24 hours or three days to investigate the time-dependent effect of rintatolimod. Proliferation rates were determined by measuring the total DNA count through fluorescence intensity detection using the bis-Benzimide fluorescent dye (Hoechst 33258) as a measure of cell amount per well, as previously described in detail [16]. The cell proliferation assay was repeated twice with three wells for each treatment group.

Cell migration assay

The effect of rintatolimod on cell migration was evaluated using a scratch assay method described by Liang et al. [17]. The scratch assay was repeated twice with three wells for each treatment group. After trypsinization, cells were

plated in 2 ml of medium in poly-L-lysine coated 12-wells plates and placed in a 37°C, 5% CO_2 incubator until a confluent monolayer was formed. A scratch was made using a 200 µL pipet tip by scratching the monolayer in a straight line. To remove debris, cells were washed twice with 1 ml medium containing 0.5% FBS and two times with 1 ml DPBS. After debris removal, 2 ml of medium supplemented with 0.25% FBS and 0.2 or 0.4 mg/ml rintatolimod was added. As recommended by Liang et al. [17], we minimized cell proliferation by using a lower percentage of serum in the growth media yet sufficient to prevent cell death. The ability of cells to migrate into the scratch area was assessed by comparing the photomicrographs (NIKON model Eclipse Ts2-FL), Imaging software: NIS Elements (Advanced Solutions for your imaging world) v.4.60.00 (Build 1171) after 0, 4, 8, and 24 hours of 4 fixed points along the scratch area. The percentage of the non-recovered scratch area was calculated by dividing the non-recovered area after 4, 8, and 24 hours by the scratch area after 0 hours using the Automated MRI Wound Healing Tool from the ImageJ software [18].

RNA isolation and multiplexed gene expression analysis

Cells treated with 0.4 mg/ml rintatolimod, 0.4 mg/ml vehicle-treated control cells, and untreated cells were snap-frozen and stored at -80°C after the proliferation assay was terminated (three days). RNA was isolated using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany). The quantity and quality of RNA samples were examined using the BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA). RNA concentration was corrected based on the percentage of fragments > 300 bp of each sample. A total of 200 ng/µl was used for hybridization using a custom-created NanoString panel that contained 76 genes known to be involved in the TLR signaling pathway. Hybridization was performed for 17 hours at 65°C, following the manufacturer's instruction (Nanostring® Technologies, Inc., Seattle, WA). The number of copies of each gene was counted by scanning 490 Fields of view using the nCounter® FLEX system (Nanostring® Technologies). The gene expression data were analyzed using the nSolver software (v.4.0). The relative expressions of genes were calculated; gene expressions in the rintatolimod-treated sample with a Log_2 fold of change (FOC) < -1 and > 1 relative to the FOC of the vehicle-treated control sample, were considered significantly differentially expressed. Pathway enrichment analysis on differentially expressed genes and analysis of transcription regulatory networks were performed using Metascape [19].

TLR-3 immunohistochemistry (IHC) staining

To further explore the TLR-3 expression pattern in PDAC and to validate the TLR-3 mRNA expression pattern, thirteen Formalin-Fixed Paraffin-Embedded (FFPE) PDAC tissue samples retrieved after Whipple's resections and three hPDAC cell lines and were stained for TLR-3. IHC staining was performed with the automated, validated, and accredited staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucson, AZ, USA) with the optiview universal DAB (diaminobenzidine) detection Kit (#760-700). The deparaffinized human tissue samples and the hPDAC cell line cytospin samples were incubated for 120 minutes at 37°C with the TLR-3 monoclonal antibodies at a dilution of 1:100 (catalog number: MA5-16184; Thermo Fisher Scientific, Waltham, MA, USA) and 1:50 (catalog number: NBP2-24875; Novus Biologicals, Denver, CO, USA), respectively. The counterstain was done by hematoxylin II counterstain for 12 minutes and a blue coloring reagent for 8 min. Each slide contained a fragment of Fresh frozen tonsil as an on-slide positive control. The TLR-3 protein expression was evaluated in a semi-quantified manner using the modified histochemical score (H-score) [20, 21]. Four independent observers (C.W.F.v.E., D.A.M.M., H.H., and M.D.) evaluated the overall expression as well as the expression of four hot-spot areas. The H-score was calculated by multiplying the percentage of stained tumor cells (0%, 25%, 50%, 75%, or 100%) by an ordinal value (0 to 3) corresponding to the intensity of the staining. The reported H-score was calculated using the median of the H-scores of all areas and was classified into four grades (1 = absent expression, 2 = weak, 3 = moderate, and 4 = strong).

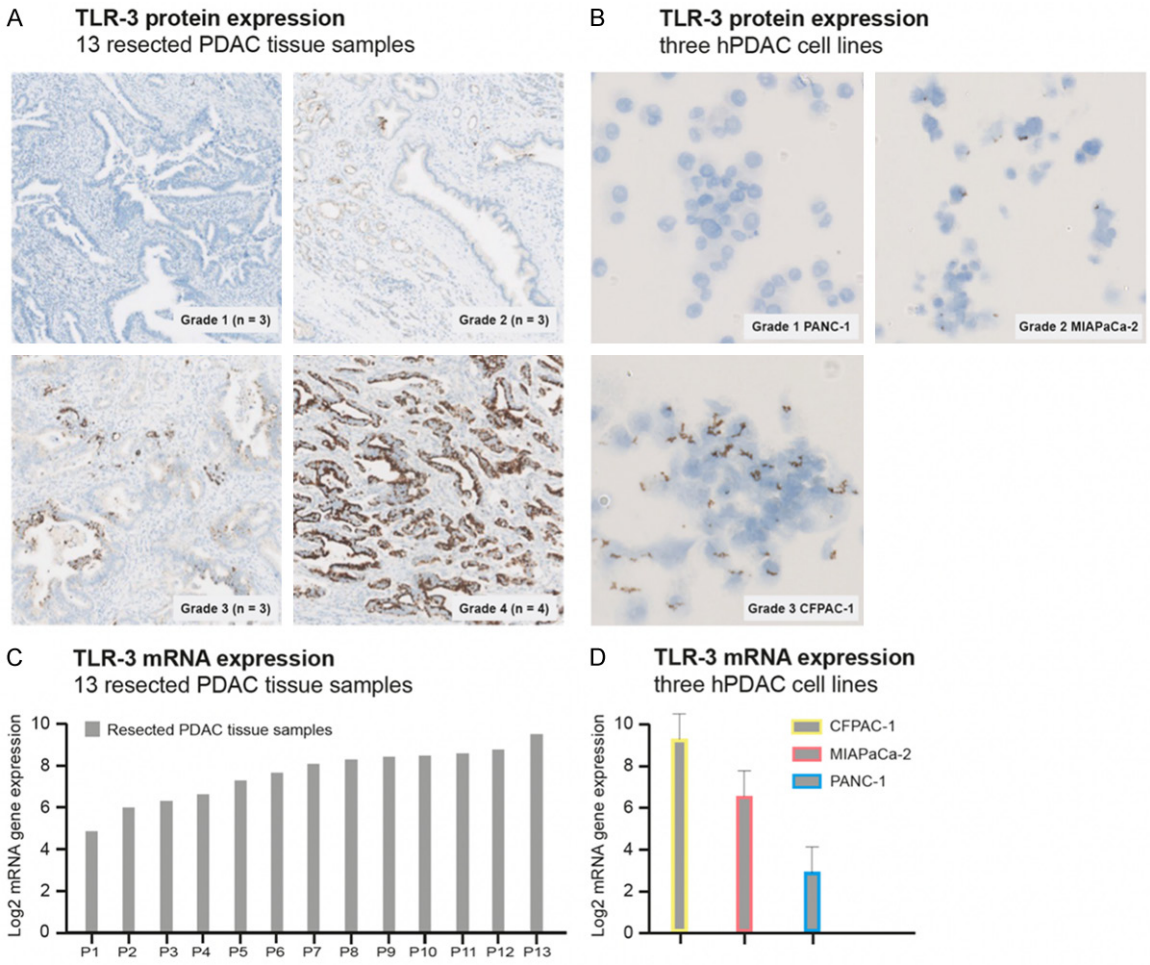

Figure 1. The heterogeneous TLR-3 protein and mRNA expression patterns in PDAC. A: Microscopic images of the TLR-3 IHC staining pattern in 13 resected PDAC tissue samples: grade 1 (absent, n = 3), grade 2 (weak, n = 3), grade 3 (moderate, n = 3), grade 4 (strong, n = 4). B: Microscopic images of TLR-3 IHC staining pattern in three human cell lines: grade 1 (absent, PANC-1), grade 2 (weak, MIAPaCa-2), grade 3 (moderate, CFPAC-1). C: Bar plots of mRNA TLR-3 expression (log_2), in which each bar represents one resected PDAC sample. D: Bar plots of mRNA TLR-3 expression (log_2) in three hPDAC cell lines, CFPAC-1 (yellow) shows high, MIAPaCa-2 (red) shows moderate, and PANC-1 (blue) shows an undetectable TLR-3 gene expression.

Statistical analysis

Measurements were analyzed using the one-way Analysis of Variance (ANOVA) test and Levene's test for equality of variances. Tukey honestly significance difference posthoc analysis was used for multiple comparisons with equal variances and unequal sample sizes. Welch's ANOVA (robust test for equality of means) was used for unequal variances, followed by the Games-Howell post hoc analysis for multiple comparisons with unequal variances and unequal samples. All tests were performed at the *P* value < 0.05 significance level. All statistical analyses were performed with SPSS version 28.0.1.

Results

TLR-3 mRNA and protein expression in tissue samples and cell lines of hPDAC

IHC analysis showed a heterogenous expression pattern of the TLR-3 protein in resected PDAC tissue samples and three hPDAC cell lines. Three hPDAC tissue samples were classified as grade 1, three as grade 2, three as grade 3, and four samples as grade 4 (**Figure 1A**). The hPDAC cell lines showed a moderate TLR-3 staining intensity in CFPAC-1, a weak intensity in MIAPaCa-2, and absent staining in PANC-1 (**Figure 1B**). In addition, multiplexed gene expression analysis revealed a compara-

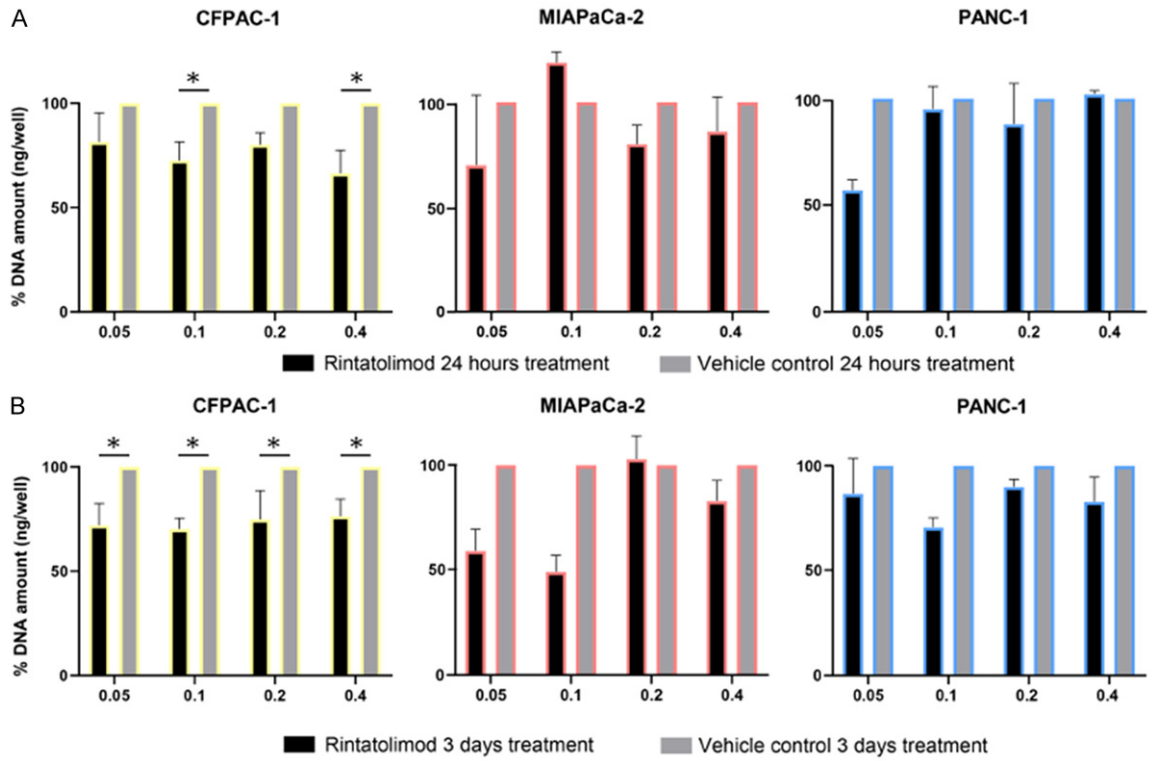

Figure 2. The effect of rintatolimod treatment on DNA counts (ng/well) representing cell proliferation in three human PDAC cell lines. Bars depict DNA counts following 24 hours (A) and three days (B) of treatment with rintatolimod (black bars) and vehicle control (grey bars) using increasing concentrations of 0.05, 0.1, 0.2, and 0.4 mg/ml. DNA counts represent the mean DNA counts (ng/well) of three wells per treatment group of the two independent experiments. Rintatolimod treatment effects on proliferation are visualized as a percentage of difference (decrease or increase) relative to vehicle control set at 100%. CFPAC-1 (yellow), MIAPaCa-2 (red), and PANC-1 (blue). *P value < 0.05.

ble heterogeneous TLR-3 mRNA expression in PDAC tissue samples (**Figure 1C**) and three hPDAC cell lines (**Figure 1D**). Only CFPAC-1 and MIAPaCa-2 cells expressed the TLR-3 gene. CFPAC-1 showed the highest mRNA expression, followed by MIAPaCa-2, while no expression was detected in PANC-1.

Cell proliferation after rintatolimod treatment

To evaluate the effect of rintatolimod on cell proliferation, we determined the amount of DNA (as a measure of cell number) in three hPDAC cell lines after 24 hours and three days of rintatolimod treatment (dose ranging between 0.05, 0.1, 0.2, and 0.4 mg/mL). Rintatolimod treatment had no significant effect on MIAPaCa-2 and PANC-1 cell growth, compared to vehicle-treated control cells (**Figure 2**). However, compared to vehicle-treated control cells, CFPAC-1 cell growth was significantly inhibited by a 24-hour treatment with 0.1-0.4 mg/mL rintatolimod (P < 0.05) (**Figure 2A**) and by 0.05, 0.1, 0.2, and 0.4 mg/mL rintatolimod during a 3-day incubation (P value < 0.05) (**Figure 2B**).

Cell migration after rintatolimod treatment

To determine basal cell migration rates and the effect of rintatolimod on wound closure (as a measure of cell migration) were investigated in three hPDAC cell lines. The basal wound closure after 4, 8, and 24 hours was the highest in PANC-1 cells and the lowest in MIAPaCa-2 cells (**Figure 3A**). No statistically significant differences (P value > 0.05) in wound closure were observed between rintatolimod and vehicle-treated control cells in any of the cell lines. Nonetheless, rintatolimod-treated CFPAC-1 cells showed a profound reduction in wound closure after 24 hours (**Figure 3B**). The 24-hour wound closure of rintatolimod and vehicle-treated control cells was 24% compared to

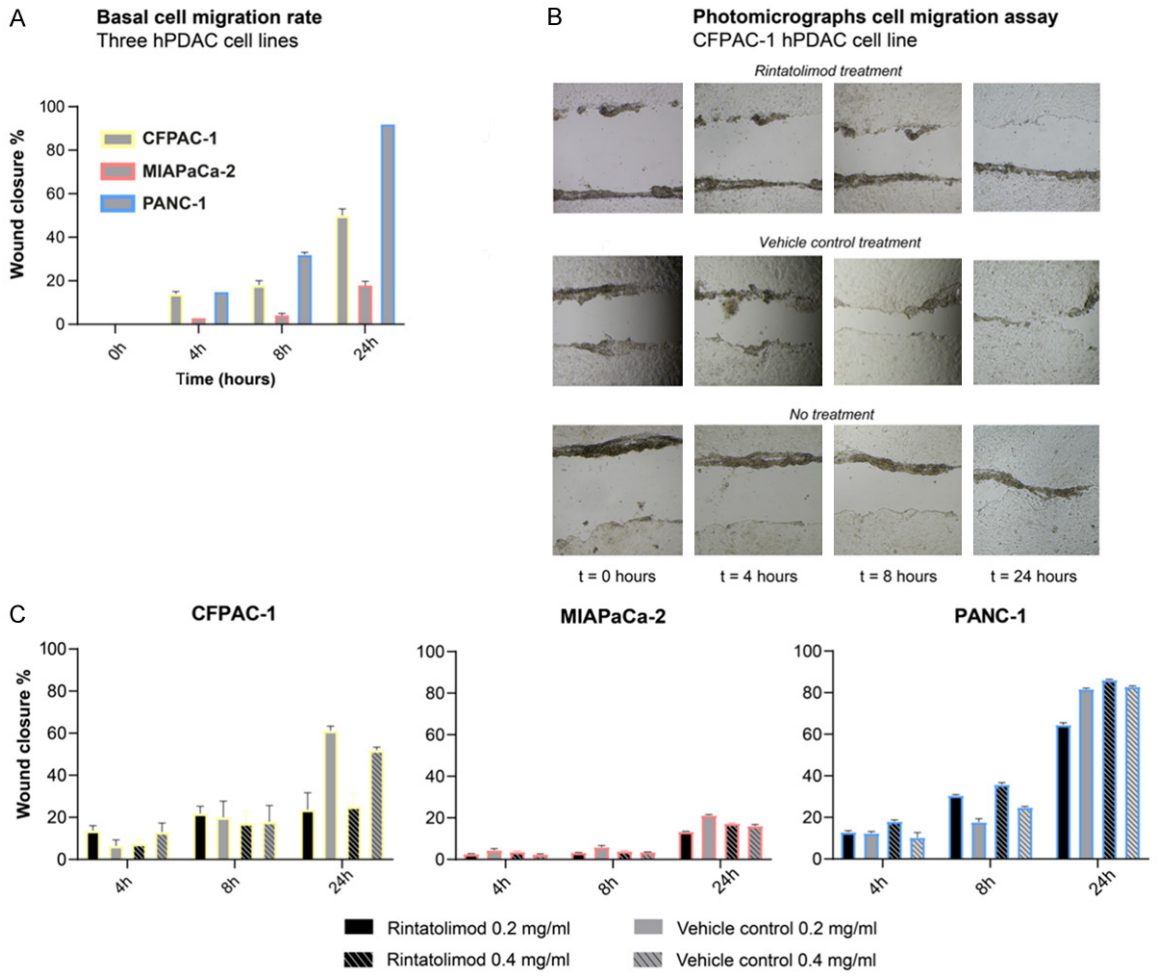

Figure 3. The effect of rintatolimod on cell migration of the three human PDAC cell lines. A: Basal cell migration after 0, 4, 8, and 24 hours. B: Photomicrographs of migration assay after 0, 4, 8, and 24 hours in untreated, 0.4 mg/mL vehicle-treated control, and 0.4 mg/mL rintatolimod-treated CFPAC-1 cells. C: The effect of 0.2 and 0.4 mg/mL rintatolimod and vehicle control treatment on cell migration after 4, 8, and 24 hours. The percentage of wound closure was calculated by dividing the unclosed wound area by the unclosed wound area at t = 0 hours. Values are expressed as the mean ± SD of two independent experiments. CFPAC-1 (yellow), MIAPaCa-2 (red), and PANC-1 (blue).

61% following 0.2 ml/mg treatment, and 25% compared to 52% following 0.4 ml/mg treatment, respectively (**Figure 3C**).

Multiplexed gene expression analysis after rintatolimod treatment

We evaluated the regulation of genes involved in the TLR-3 signaling pathway following a three-day treatment with the TLR-3 agonist rintatolimod using multiplexed gene expression analysis and a custom-created 76-gene panel. Cells of three hPDAC cell lines were untreated, 0.4 mg/mL vehicle-treated, or 0.4 mg/mL rintatolimod-treated. We only investigated the gene expression profiles of 0.4 mg/mL vehicle-treated control or 0.4 mg/ml rintatolimod-treated cells due to the results of the proliferation and migration assays. No genes with a Log_2 |FOC > 1.0| were identified between untreated and 0.4 mg/mL vehicle-treated control cells. Comparison between 0.4 mg/mL rintatolimod-treated and 0.4 mg/mL vehicle-treated control cells revealed fifteen altered genes in CFPAC-1 cells, six altered genes in MIAPaCa-2 cells, and one altered gene in PANC-1 cells with a Log_2 |FOC > 1.0| (**Figure 4A**). Specifically, CFPAC-1 cells showed a Log_2 FOC > 1.0 upregulation in gene expression of genes related to the CXCL family (1, 2, 3, 5, 6, 10, and 11), IFIH1, IFIT1 and 2, IFNL2, IL6 and 32, LTB, and TLR1. A Log_2 FOC < -1.0 downregu-

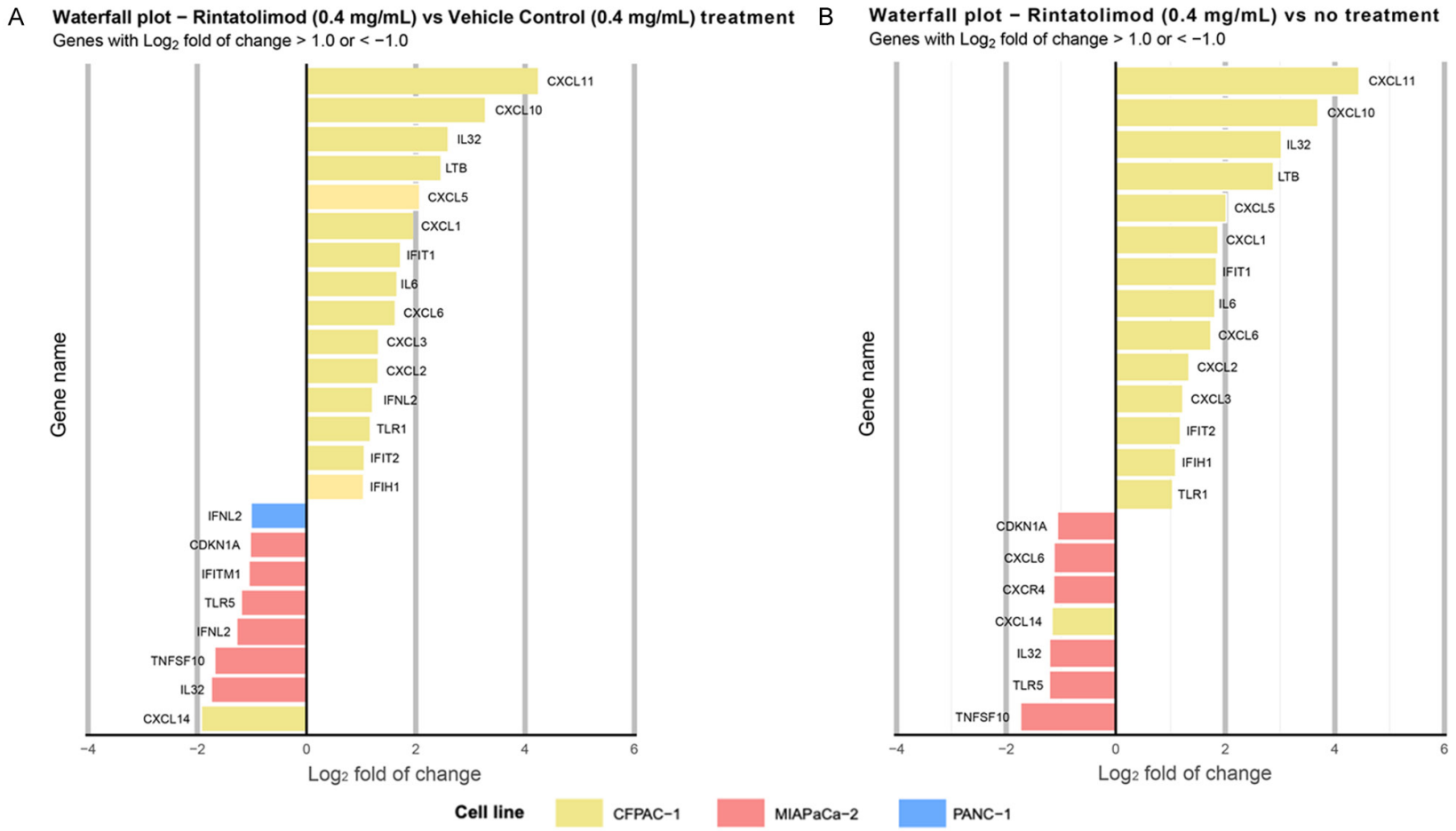

Figure 4. Waterfall plot displays gene expression alterations after 0.4 mg/mL rintatolimod treatment compared to 0.4 mg/mL vehicle control (A) and no (B) treatment in three human PDAC cell lines (CFPAC-1 (yellow), MIAPaCa-2 (red), and PANC-1 (blue)). Only genes with a gene expression alteration of Log_2 fold of change ≥ |1.0| are included.

Table 1. Gene expression alterations after 0.4 mg/mL rintatolimod treatment compared to 0.4 mg/mL vehicle control treatment in three human PDAC cell lines (CFPAC-1, MIAPaCa-2, and PANC-1)

Gene name	CFPAC-1 Log_2 FOC*	MIAPaCa-2 Log_2 FOC*	PANC-1 Log_2 FOC*
CDKN1A	0.540	-1.03	0.020
CXCL1	1.95	-0.139	-0.162
CXCL10	3.28	< Limit of detection	< Limit of detection
CXCL11	4.23	< Limit of detection	< Limit of detection
CXCL14	-1.93	< Limit of detection	< Limit of detection
CXCL2	1.31	-0.799	0.061
CXCL3	1.32	-0.725	0.362
CXCL5	2.07	-0.351	< Limit of detection
CXCL6	1.63	-1.11	0.183
IFIH1	1.05	0.083	0.280
IFIT1	1.72	-0.436	0.418
IFIT2	1.06	-0.744	0.004
IFITM1	0.270	-1.06	-0.209
IL32	3.03	-1.21	-0.520
IL6	1.66	< Limit of detection	< Limit of detection
LTB	2.47	-0.591	-0.709
TLR1	1.17	-0.004	0.831
TLR5	-0.770	-1.20	-0.153
TNFSF10	0.96	-1.65	0.423

Genes with a gene expression alteration of Log_2 fold of change ≥ |1.0| in any of the cell lines are included. *FOC: fold of change.

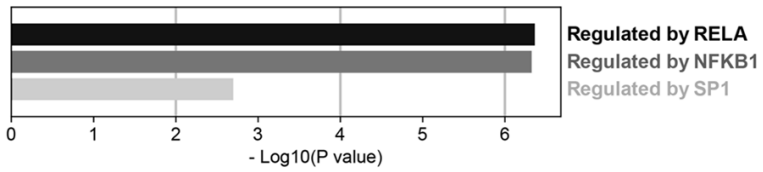

Figure 5. Summary of enrichment analysis (transcriptional regulatory networks in TRRUST) in metascape of 14 differentially expressed genes (up-regulated) in CFPAC-1 cells. Three transcription factors were identified. Log_{10} (*P* value) is the *P* value in log base 10.

lation in gene expression was observed among CXCL14 in CFPAC-1 cells, CDKN1A, IFITM1, IFNL2, IL32, TLR5, and TNSF10 in MIAPaCa-2 cells, and IFNL2 in PANC-1 cells (**Table 1**). In addition, a comparison between 0.4 mg/mL rintatolimod-treated and untreated cells showed identical results except for the IFNL2 gene, which was not altered with a Log_2 |FOC > 1.0| in all cell lines (**Figure 4B**). The sixteen altered genes in CFPAC-1 cells were subjected to pathway enrichment analysis to investigate their relation to the TLR-3 signaling pathway. Three transcription factors, NFKB1, RELA, and SP1 were significantly associated with the differentially expressed genes (**Figure 5**) and are involved in the TLR-3 pathway as reviewed in the Reactome Knowledge database [22]. A schematic overview of the TLR-3 signaling pathway is depicted in **Figure 6**.

Discussion

Immune-modulating TLR-3 agonists are considered a promising novel treatment option for pancreatic cancer and might have additional anti-tumoral effects on epithelial tumor cells expressing TLR-3. This theory is based on previously reported associations of TLR-3 signaling with cell cycle regulation and migration pathways in various human cancer cells [23-32]. This study evaluated the direct anti-tumoral effects of the TLR-3 agonist rintatolimod in three hPDAC cell lines in the absence of immune cells. We hypothesized that TLR-3 activation affects cell proliferation as well as cell migration and that the abundance of TLR-3 expression determines the extent of the effect. First, we investigated the TLR-3 protein and mRNA expression in human resected tumor samples and observed a heterogeneous expression that was both intra- and extracellularly present. Thereafter, we determined the TLR-3 protein and mRNA expression in three human pancreatic cell lines and found a corresponding heterogeneous expression pattern (high in CFPAC-1, moderate in MIAPaCa-2, and undetectable in PANC-1). After treating these three cell lines with rintatolimod, we observed significantly lower cell numbers (amount of DNA per well, representing proliferation) and lower migration rates only in the high TLR-3 expressing CFPAC-1 cells. This indeed suggests that the variating TLR-3 abundance of our three hPDAC cell lines influences the effect of rintatolimod on cell proliferation

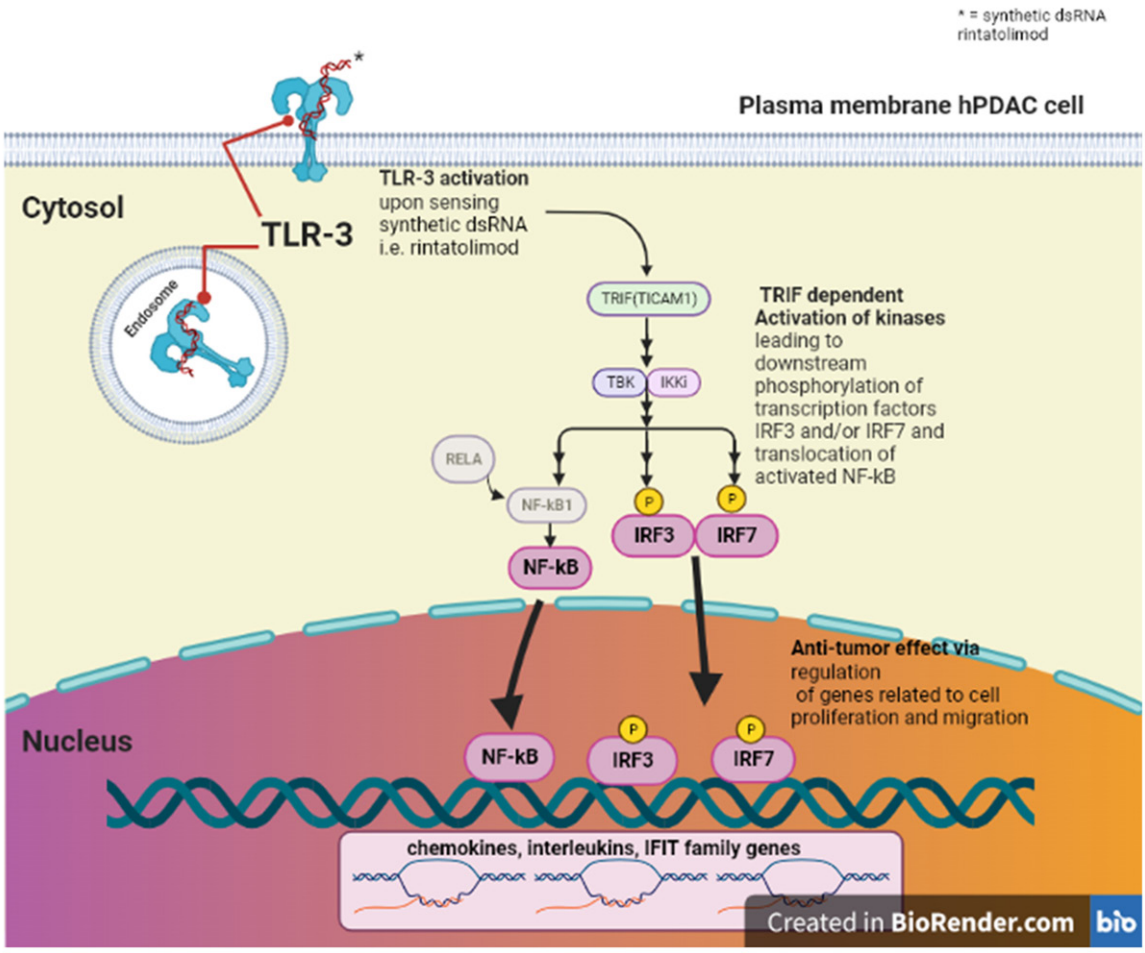

Figure 6. Schematic overview of the TLR-3 signaling pathway.

and cell migration. Finally, multiplexed gene expression analysis showed significantly upregulated genes in CFPAC-1 cells, and pathway gene enrichment analysis identified transcription regulatory networks of these genes within the TLR-3 pathway, indicating a direct receptor-bound activation.

The crosstalk between TLR-3 activation and (cancer) cell proliferation and migration is not exactly known. In pancreatic cancer cells, proliferation and migration are mediated via key signaling pathways. Including the PI3K/AKT/p53 axis that is primarily involved in malignant cell proliferation as well as cell motility, and the ERK and Ras/RAF/MAP kinase signaling pathways that are linked to overall cell survival as well as cell motility [33]. The activation of the TLR-3 cascade is known to result in i: TICAM1-dependent programmed cell death, ii: TAK1-dependent IKK and NF-kappa-B activation and interferon production, and iii: MAP kinase activation. Studies investigating different epithelial cancer cells suggest that TLR-3 signaling could affect cell proliferation via the inactivation of the PI3K/Akt/p53 pathway [23, 24, 28, 29]. Harashima et al. showed that TLR-3 activation using poly(I:C) triggered apoptosis and growth arrest in prostate cancer cells, along with regulation of tumor suppressor gene p53, downstream of Akt and cyclinD1 gene involved in malignant cell cycle G1 phase progression [29]. Unfortunately, we could not associate the underlying mechanism here with the PI3K/Akt/p53 pathway because our custom-created panel did not include target genes involved in this pathway. Nevertheless, multiplexed mRNA expression analysis showed an upregulation of various genes associated with regulating transcription factors NFKB1 and RELA which are known to be involved in the TLR-3 signaling pathway. Furthermore, in rintatolimod-treated

CFPAC-1 cells, we observed upregulated genes encoding for chemokines CXCL-10 and -11, interleukin IL-32, and IFIT (IFN-Induced proteins with Tetratricopeptide repeats) that have been associated with cell proliferation and cell migration in previous work [34-37].

We evaluated in vitro cell migration using a scratch assay method previously validated with the Cytoselect Wound Healing assay [38]. As recommended by Liang et al. (20), we minimized the effect of cell proliferation on our cell migration assay by analyzing migration rates 8 and 24 hours after the scratch, and we used a lower percentage of serum in the growth media that was yet sufficient to maintain cell survival. Although not statistically significant, a decrease in cell migration after rintatolimod treatment was observed only in CFPAC-1 cells with high TLR-3 expression. The observed trend of a low migration rate in cells with increased TLR-3 expression is in line with previous literature that showed reduced migration after treatment with TLR-3 agonists in lung carcinoma cells, hepatocellular carcinoma cells, and oral squamous carcinoma cells expressing TLR-3 [26, 30, 31, 39]. Specifically, reduced cell motility was explained by TLR-3-induced tumor suppressor activity of tumor suppressor phosphatase and tensin homolog (PTEN). Treatment with polyI:C induced upregulation of PTEN and inhibition of Akt phosphorylation. [39]. The tumor-suppressor activity of PTEN has extensively been documented [40-42], and upregulation of PTEN has been associated with decreased migration capacity in various cancers [39, 43-45]. Our results warrant further research in downstream signaling pathways, cell growth, and cell motility linked to TLR-3 activation.

Rintatolimod has undergone extensive pre-clinical and clinical safety testing and has been administered to patients in doses ranging from 200 mg to 1200 mg. Based on the recommended clinically effective dose of 6 mg/kg infused over 30 minutes, rintatolimod has a C_{max} of 74 ug/ml therefore using in vitro concentrations in the range that was used here is well within the achievable blood concentration of rintatolimod in humans [46, 47]. Noteworthy, the investigated direct anti-tumoral effects of rintatolimod in vitro could be accompanied by indirect anti-tumoral properties in vivo. Rintatolimod was reported to induce various immunomodulatory effects potentially favorable in the treatment of pancreatic cancer, including dendritic and T cell maturation, increasing peripheral B cell abundance, and inducing (adaptive) immune responses [15, 48, 49].

Our study has some limitations. Firstly, only three hPDAC cell lines were included in this study. Nevertheless, our cell lines covered the heterogeneous TLR-3 expression pattern in PDAC. Secondly, we did not investigate the phosphorylation status of IRF3 after rintatolimod treatment, which is a direct indicator of TLR-3 activation. However, the ability of rintatolimod to activate TLR-3 has been extensively studied in various experimental models, and the involvement of IRF3 in the TLR-3 signaling cascade has been previously described [50-52]. Moreover, our results demonstrate that CFPAC-1 cells, which exhibit high levels of TLR-3 protein and mRNA expression, displayed a gene expression profile regulated by transcription factors NFKB1, RELA, and SP1 within the TLR-3 signaling cascade. Thirdly, the multiplex gene expression measurement was limited to a pre-selected panel of 70 genes that did not include PI3K/Akt/p53 and ERK/MAPK target genes. Hence, various downstream genes important in proliferation and migration pathways were not included. Lastly, we measured gene expressions, but functional protein analysis is lacking.

In conclusion, this study evaluates the direct effect of rintatolimod in three hPDAC cell lines in the absence of immune cells. We observed a heterogeneous protein and mRNA expression of TLR-3 in PDAC tissue samples and three hPDAC cell lines. Only in pancreatic cancer cells with high TLR-3 mRNA expression (CFPAC-1) does rintatolimod treatment reduces proliferation and migration rates. In addition, rintatolimod-treated CPFAC-1 cells showed various upregulated genes involved in the TLR-3 signaling pathway. Altogether, our findings suggest that rintatolimod (Ampligen®) is a promising agent with the potential to induce cell growth arrest and reduce cell mobility in pancreatic cancer patients with sufficient tumor levels of TLR-3. Further research is needed to validate our findings. This includes investigating a TLR-3 knockout model and evaluating downstream

target genes related to proliferation and migration pathways.

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Disclosure of conflict of interest

None.

Abbreviations

ANOVA, Analysis of Variance; CDKN1A, Cyclin Dependent Kinase Inhibitor 1A; CXCL1-2-3-5-6-10-11-14, C-X-C motif chemokine Ligand 1-2-3-5-6-10-11-14; CXCR4, C-X-C chemokine receptor 4; DPBS, Dulbecco's Phosphate Buffered Saline; dsRNA, double-stranded RNA; FBS, Fetal Bovine Serum; FFPE, Formalin-Fixed, Paraffin-Embedded; FOC, Fold Of Change; H-score, Histochemical score; IFN-I, type I interferon; IHC, Immunohistochemistry; IFIH1, Interferon Induced with Helicase C domain 1; IFIT1-2, Interferon Induced protein with Tetratricopeptide Repeats 1-2; IFITM1, Interferon Induced Transmembrane Protein 1; IFNL2, Interferon Lambda 2; IL6-32, Interleukin 6-32; IRF3, induced transcription factor IFN Regulatory Factor 3; LTB, Lymphotoxin Beta; NFKB1, Nuclear Factor Kappa Beta subunit 1; RELA, Nuclear factor-kappa Beta p65; Rintatolimod, PolyA.PolyU and Polyl:C12U or Ampligen®; SP1, Specificity Protein 1; TLR1-3-5, Toll-Like Receptor 1-3-5; TNFSF10, Tumor Necrosis Factor Superfamily member 10.

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