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CXCL9/10/11, a regulator of PD-L1 expression in gastric cancer

Chenlu Zhang^{1,2}, Zhi Li¹, Ling Xu¹, Xiaofang Che¹, Ti Wen¹, Yibo Fan¹, Ce Li¹, Shuo Wang¹, Yu Cheng¹, Xiaoxun Wang¹, Xiujuan Qu^{1*}  and Yunpeng Liu^{1*}

Abstract

Background: Programmed death-ligand 1 (PD-L1) is an immunosuppressor that plays an important role in cancer treatments. Although majority of the studies demonstrated that PD-L1 expression was regulated by cellular intrinsic and extrinsic controls, and IFN- γ was a key molecule of extrinsic control, other studies imply that other cytokines play important roles in PD-L1 expression. In this study, we investigated the regulation of PD-L1 by chemokine signaling pathway in gastric cancer (GC) cells.

Methods: Bioinformatics was used to explore the PD-L1-related genes in GC and propose a hypothesis. PD-L1 and CXCR3 expression were detected by western blot in SGC7901 and MKN74 cell lines. Meanwhile, PD-L1 and CXCR3 expressions were immunohistochemically assessed for their relevance. Moreover, PD-L1, pSTAT3 and pAkt were detected after treatment with CXCL9/10/11. Furthermore, PD-L1, pSTAT3 and pAkt were evaluated after blocking chemokine signaling in SGC7901 cells.

Results: Based on online database analysis, CXCL9/10/11-CXCR3 is proposed to upregulate PD-L1 expression by activating the STAT and PI3K-Akt pathways. This hypothesis was confirmed by *in vitro* and *in vivo* experiments. CXCR3 and PD-L1 were expressed in GC cell lines and tissues, and the expression of CXCR3 and PD-L1 was positively related. PD-L1 was upregulated after treatment with CXCL9/10/11, accompanied by activation of STAT3 and Akt. After blocking chemokine signaling, upregulation of PD-L1 and activation of STAT3 and Akt were diminished.

Conclusions: CXCL9/10/11-CXCR3 upregulated the expression of PD-L1 by activating the STAT and PI3K-Akt signaling pathways in GC cells. There was a significant positive correlation between the expression of PD-L1 and CXCR3 in gastric cancer patient tissues.

Keywords: PD-L1, CXCR3, CXCL9/10/11, Gastric cancer

Background

Although the incidence of gastric cancer (GC) has greatly reduced in developed countries, it remains the third leading cause of cancer-related deaths worldwide [1], and one of the most common cancers in Asia. In China, GC is the third leading cause of cancer-related deaths, and the proportion of advanced GC is about 70%. Although targeted drugs prolong the survival of patients with advanced GC by more than a year, the 5-year survival rate is less than 20% [2, 3]. Therefore, new treatment strategies need to be developed.

Cancer immunotherapy has shown major advancements during the past few years. Immune checkpoints such as cytotoxic T-lymphocyte-associated antigen (CTLA-4) and programmed death-ligand 1 (PD-L1) can suppress the activity of T-lymphocytes, which could recognize and eliminate cancer antigens [4]. Therefore, blocking programmed death-1 (PD-1) is now an attractive medical approach to enhance anti-tumor immunity [5–7]. In 2014, the PD-1 monoclonal antibody pembrolizumab targeting the PD-L1/PD-1 signaling pathway, had shown significant clinical effect in patients with high PD-L1 expression in advanced melanoma and non-small cell lung cancer. Moreover, it was approved by the US FDA as the first-line treatment for advanced melanoma in 2015 [8]. Recently, the clinical trial KEYNOTE-012

* Correspondence: xiujuanqu@yahoo.com; cmuliuyunpeng@hotmail.com

¹Department of Medical Oncology, the First Hospital of China Medical University, NO.155, North Nanjing Street, Heping District, Shenyang 110001, China

Full list of author information is available at the end of the article



showed a good clinical effect of pembrolizumab in PD-L1-positive advanced GC [9], which suggested that PD-L1-positive GC patients might benefit from blocking the PD-L1/PD-1 signaling pathway. Therefore, researchers and physicians have paid increasing attention to cancer immunotherapy, and the molecular target therapy of immunological checkpoints has brought new hope for solid tumors, with PD-L1 being a promising one.

Majority of the recent studies have shown that PD-L1 expression is regulated by intrinsic and extrinsic mechanisms in cancer cells. Intrinsic immunologic tolerance associates carcinogenesis with PD-L1 expression, such as overexpression of ALK and Ki-67, activation of Kras, and inactivation of PTEN and Lkb1 [10–14]. Additionally, several reports have suggested that extrinsic immunologic tolerance plays an important role in the regulation of PD-L1. Various cytokines play an important regulatory role in tumor microenvironment. Interferon- γ (IFN- γ) secreted by activated immune cells is not only an important anti-tumor factor but also a central extrinsic factor that induces upregulation of PD-L1 in tumor cells [15–17]. Recently, cytokines, such as interleukin-27 (IL-27), IL-17, tumor necrosis factor- α (TNF- α) [18, 19], etc., have been found to regulate PD-L1. Therefore, it is important to elucidate the regulatory mechanism between cytokines and PD-L1.

In the present study, bioinformatics was used to explore the PD-L1-related genes in GC and propose a hypothesis. In vitro experiments were conducted to confirm the hypothesis, which clarified that CXCL9/10/11-CXCR3 upregulated PD-L1 expression by activating the STAT and PI3K-Akt pathways in SGC7901 and MKN74 GC cell lines. These findings suggest a novel mechanism regulating the immune-evading factor PD-L1.

Methods

GSEA analysis

RNASEQV2 dataset was obtained from the Cancer Genome Atlas (TCGA) (<https://gdc-portal.nci.nih.gov/>). Another two mRNA expression profiles (GSE15459 and GSE62254) were downloaded from Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>). Above two datasets were acquired using Affymetrix Human Genome U133 Plus 2.0 Array. TCGA dataset included surgically resected 375 GC samples with complete data. Dataset GSE15459 included surgically resected 192 GC samples with complete data from the Singapore patient cohort [20]. Dataset GSE62254 contained surgically resected 300 GC samples with complete data from the Korean patient cohort [21].

The TCGA data was preprocessed according to the Bioconductor/TCGABiolinks function package flow. The gene expression profile data were extracted using the Bioconductor/GEOquery function package. Gene CD274

corresponds to two probes, “223834_at” and “227458_at”, and subsequent analysis was performed on the above two probes. The expression of CD274 in the samples was sorted from high to low, with the sample whose signal above the median value as the high expression group and below the median as the low expression group.

The GSE62254, GSE15459 and TCGA-STAD-RNA SEQV2 data were substituted into GSEA2.0.14 to analyze the effect of high and low expression level of CD274 on various pathways of biological pathways, and the reference gene set was set to the c2.cp.kegg.v5.1.symbols.gmt gene set in the MsigDB database of the GSEA website. The analysis was repeated 1000 times for each one according to the default weighted enrichment statistic method. In the GSEA, based on the condition of $P < 0.05$ and false discovery rates (FDR) < 0.25 , the first 20 pathways were ranked as the “significant enrichment genes” according to the FDR value from low to high, if $P = 0$ and FDR = 0, the pathways should be included. The software R 3.2.3 was used to perform all the statistical analyses.

Functional enrichment analysis

Based on TCGA dataset, the correlation coefficient r between CD274 and the expression of each gene was calculated by Spearman correlation analysis. Based on the condition of $r > 0.6$, genes were selected for further analysis. The database for annotation, visualization and integrated discovery (DAVID) was used to analyze list of genes derived from high-throughput genomic experiments. DAVID online tool [22] for Gene functional classification was used to perform the enrichment analysis of the biological processes of CD274 related genes in order to identify the enriched pathways and genes [23].

Reagents and antibodies

CXCL9/10/11 were purchased from Pepro Tech (USA). The PD-L1 antibody was purchased from Cell Signalling Technology (USA). The CXCR3 antibody was purchased from ABCAM (USA). The CXCR3 antagonist AMG487 was purchased from Tocris (USA). The PI3K/AKT inhibitor LY294002 and the STAT3 inhibitor STATIC were obtained from Sigma (St. Louis, MO, USA). The anti-STAT3, anti-pSTAT3, anti-Akt, anti-pAkt, anti-ERK, anti-pERK, and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology (USA).

Cells and cell culture

Human gastric cell lines SGC-7901 and MKN74 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (China). Cells were grown in RPMI 1640 (Rosewell Park Memorial Institute) containing with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C [24].

Western blot analysis

Cells were seeded at 2×10^5 per well in 6-well plates and incubated overnight; Cells were treated with CXCL9/10/11 (100 ng/ml) for indicated times. Cells were lysed in lysis buffer. The method of Western blot was discussed in our previous study [25].

Small interfering RNA transfections

The siRNA sequences were from View solid biotechnology co., LTD (Beijing, China). The AKT siRNA sequence was 50-CUCACAGCCCUGAAGUACUtt-30. The siRNAs were

transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer’s instructions.

Patients and tissue specimens

The files of 92 patients who underwent surgical resection of gastric cancer between 2006 and 2011 at the First Hospital of China Medical University, were included in our study. Patient information included age, gender, TNM stage, histopathologic type and metastases. All patients were not treated with any chemotherapy. This study was approved by the Human Ethics Review Committee of

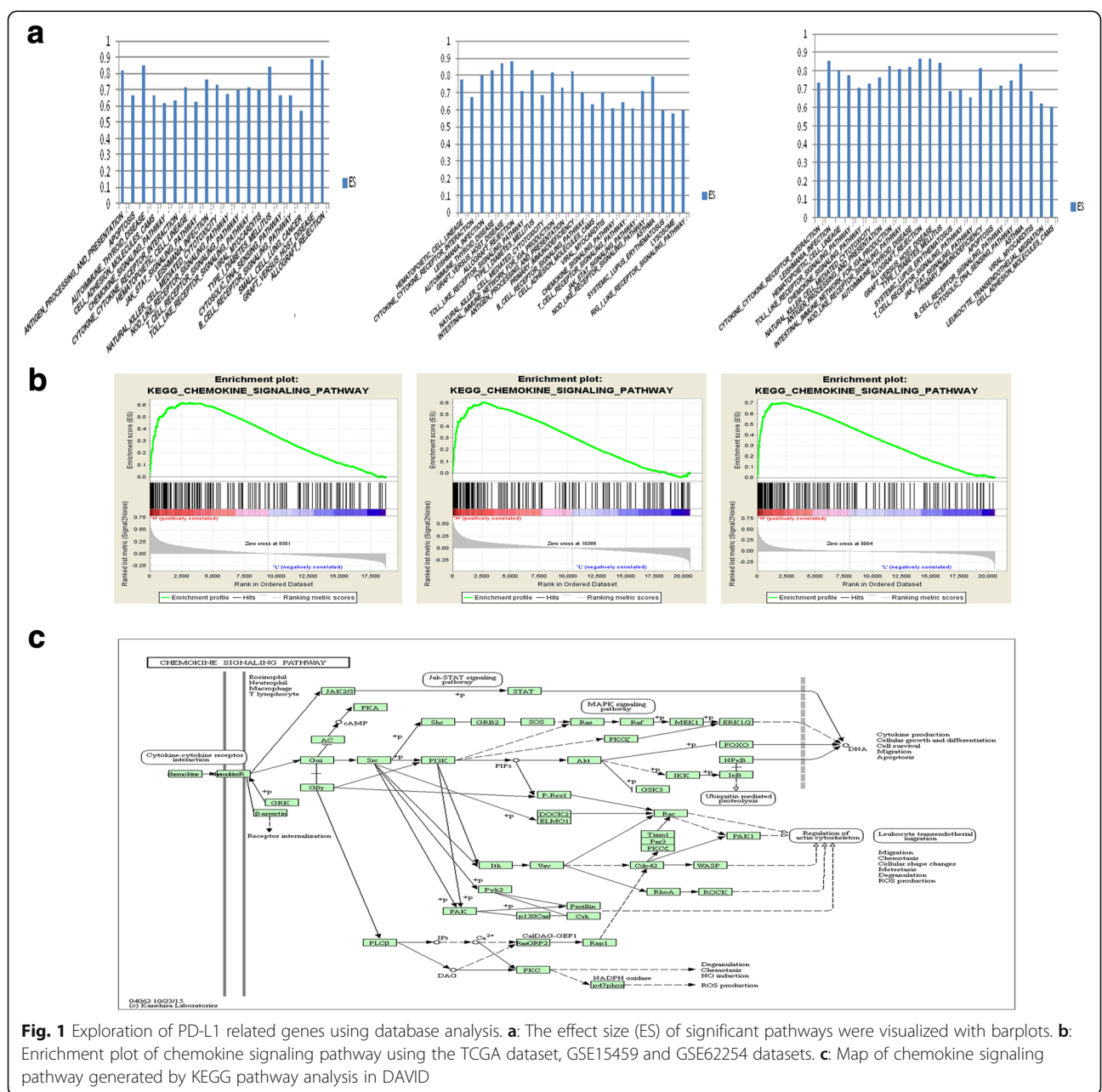


Fig. 1 Exploration of PD-L1 related genes using database analysis. **a:** The effect size (ES) of significant pathways were visualized with barplots. **b:** Enrichment plot of chemokine signaling pathway using the TCGA dataset, GSE15459 and GSE62254 datasets. **c:** Map of chemokine signaling pathway generated by KEGG pathway analysis in DAVID

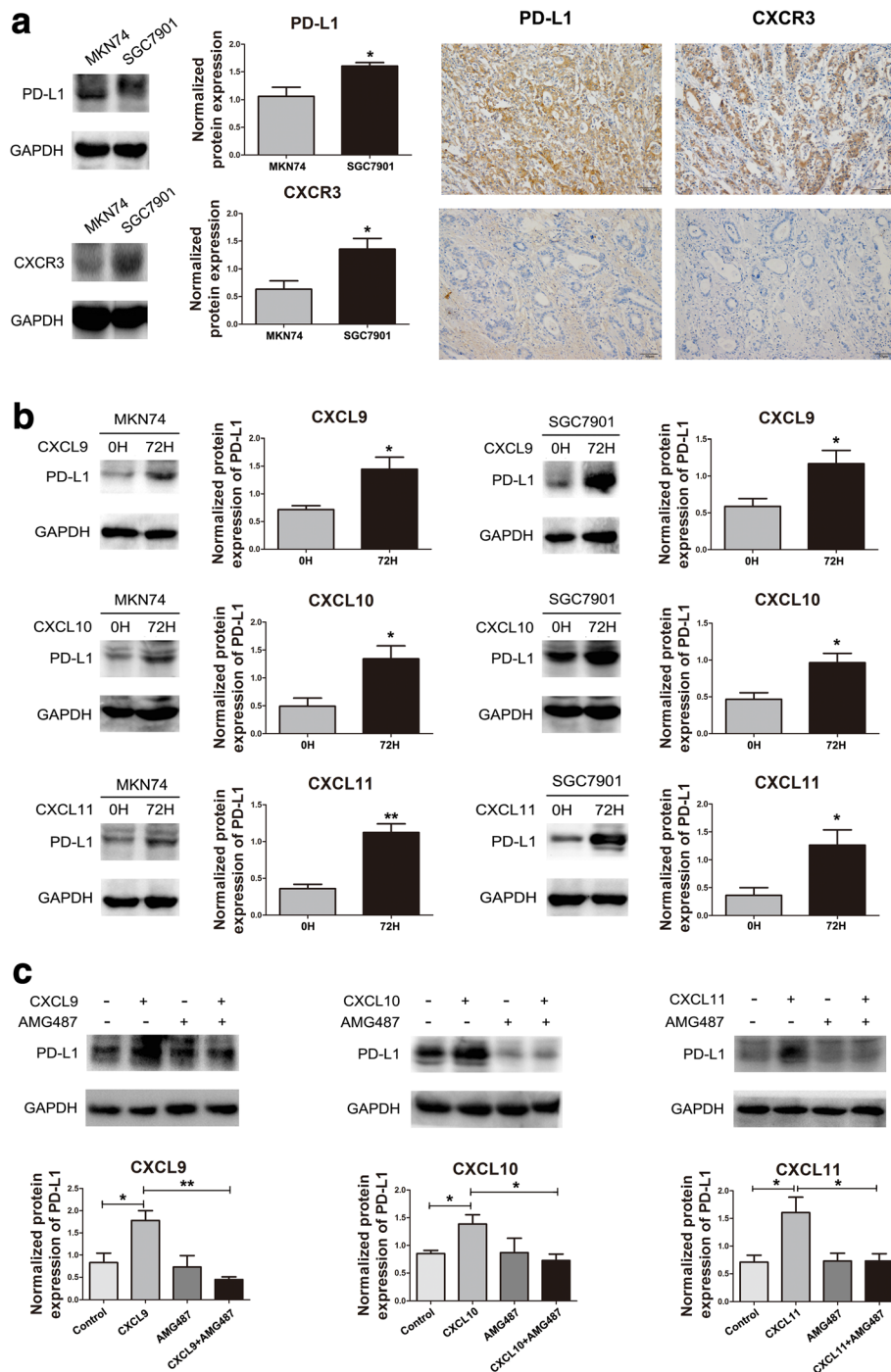


Fig. 2 CXCL9/10/11 upregulated PD-L1 expression in gastric cancer cells and tissues. **a:** CXCR3 and PD-L1 expression were analyzed by Western blot and IHC. Two representative cases from 92 clinical gastric cancer specimens were shown. The gene expression level was evaluated in three random visual fields. Original magnifications: 200x. **b:** SGC7901 and MKN74 cells were incubated with CXCL9/10/11 (100 ng/mL) for 72 h, the PD-L1 expression was analyzed by Western blot. **c:** SGC7901 cells were pretreated with or without AMG487 (1 μM) for 2 h followed by CXCL9/10/11 (100 ng/mL) stimulation for 72 h. Cell lysates were collected for Western blot analysis. Normalized protein expression levels were calculated and analyzed. The gels were run under the same experimental conditions. The band intensities were calculated using the ImageJ 1.46r software. GAPDH was used as an internal control for the total protein measurement. The ratio of the target gene to GAPDH was used to conduct the statistical analysis. * $P < 0.05$ and ** $P < 0.01$, as determined by Student's t-test

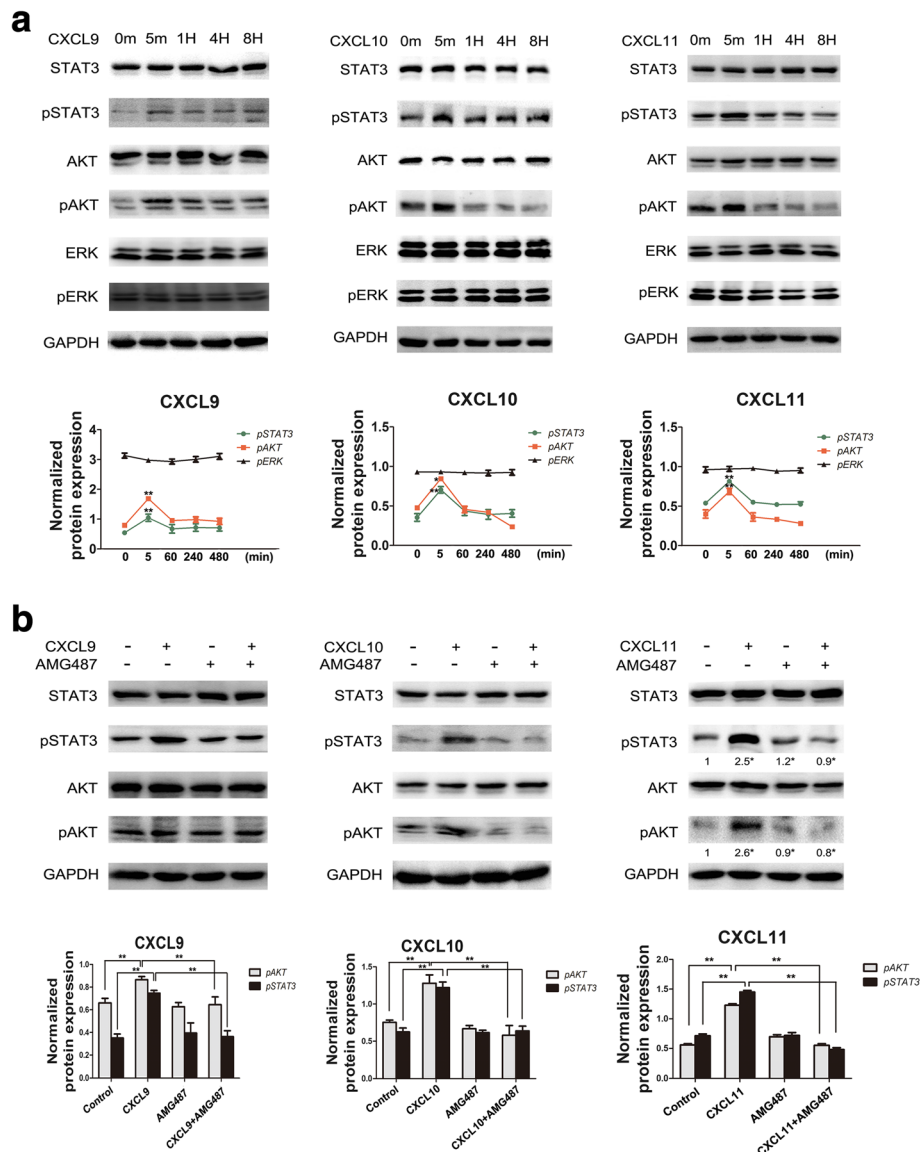
China Medical University; informed consent was obtained from all patients.

Immunohistochemistry and evaluation

Immunohistochemical staining was performed using a streptavidin-peroxidase procedure. CXCR3 and PD-L1 expression were investigated using antibodies against CXCR3 (Abcam, USA) and PD-L1 (Cell Signalling Technology, USA). All other reagents were from Sigma.

The specificity of all antibodies was confirmed by western blotting.

PD-L1 and CXCR3 were detected by immunohistochemistry (IHC) as described previously. Staining was graded into four categories: 0 (staining equal to or less than 5%), 1+ (staining of 6–25%), 2+ (staining of 26–50%), 3+ (staining more than 50%). Tissues with scores of 2+, or 3+ were considered to be positive [26]. All histological and IHC slides were evaluated by a certified surgical pathologist in our department.



Statistical analysis

All statistical tests were performed using SPSS (Statistical Package for the Social Sciences) 17.0 computer software. Differences between two groups were evaluated by Student's t-test. A P-value of 0.05 or less was considered to indicate a statistically significant difference. Spearman correlation analysis was used to determine the correlation of PD-L1 and CXCR3 expression.

Results

The hypothesis that CXCL9/10/11-CXCR3 signaling upregulated PD-L1 expression was established by bioinformatics

GSEA analysis

Using the TCGA, GSE15459 and GSE62254 datasets, the pathways ranked as the “significant enrichment genes” were shown in Fig. 1a, b and Additional file 1: Table S1,

Additional file 2: Table S2, Additional file 3: Table S3. We found the “CHEMOKINE_SIGNALING_PATHWAY” was ranked relatively high, and was related to both tumor and immune system.

Functional enrichment analysis

A total of 81 genes that were closely related to PD-L1 were selected by Spearman correlation analysis (Additional file 4: Table S4). The 81 genes were mapped to the DAVID database to investigate the functional pathways. After analysis of Gene functional classification module in DAVID database, interestingly, CXCL9/10/11 and CCL4 were grouped together (Enrichment Score: 6.15), and the remaining genes were clustered together (Additional file 5: Table S5). The chemokine signaling pathway and PD-L1 appeared to be closely related. CXCL9/10/11 are members of ELR-negative CXC chemokine subfamily, and CXCR3 is their co-

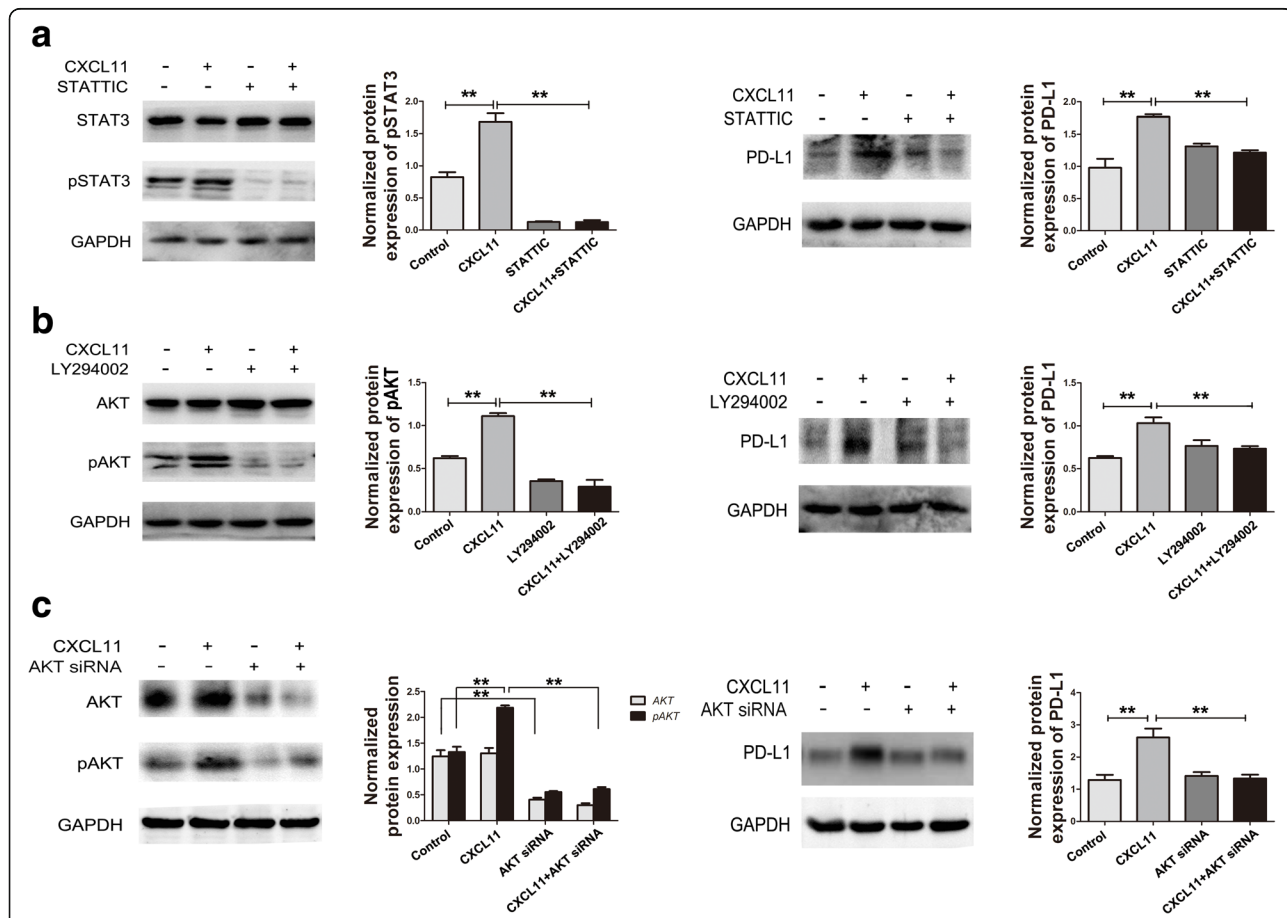


Fig. 4 CXCL11 induced PD-L1 upregulation and the activation of STAT3 and Akt. **a:** SGC7901 cells were pretreated with or without STAT3i (5 μM) overnight followed by CXCL11 (100 ng/mL) stimulation for 5 min and 72 h, the phosphorylation of STAT3 and PD-L1 were analyzed by Western blot. **b:** SGC7901 cells were pretreated with or without LY294002 (25 μM) for 2 h followed by CXCL11 (100 ng/mL) stimulation for 5 min and 72 h, the phosphorylation of Akt and PD-L1 were analyzed by Western blot. **c:** SGC7901 cells were pretreated with or without AKT siRNA for 24 h, followed by CXCL11 (100 ng/mL) stimulation for 5 min and 72 h, the phosphorylation of Akt and PD-L1 were analyzed by Western blot. Normalized protein expression levels were calculated and analyzed. The gels were run under the same experimental conditions. The band intensities were calculated using the ImageJ 1.46r software. GAPDH was used as an internal control for the total protein measurement. The ratio of the target gene to GAPDH was used to conduct the statistical analysis. *P < 0.05 and **P < 0.01, as determined by Student's t-test

receptor. Therefore, we could conjecture that CXCL9/10/11-CXCR3 signaling was closely related to PD-L1. According to the map of chemokine signaling pathway generated by KEGG pathway analysis in DAVID (Fig. 1c), STAT, Akt and ERK signaling pathways act as its downstream pathways. STAT, Akt and ERK are known to participate in the regulation of PD-L1 expression [27, 28]. Thus, we hypothesized CXCL9/10/11-CXCR3 could upregulate PD-L1 expression by activating STAT, PI3K-Akt and ERK pathways.

CXCL9/10/11-CXCR3 upregulated PD-L1 expression by activating the STAT and PI3K-Akt pathways in gastric cancer cells and tissues

We previously performed bioinformatics and found that CXCL9/10/11-CXCR3 might upregulate PD-L1 expression by activating the STAT, PI3K-Akt and ERK pathways, however, in order to verify the above hypothesis, we performed a series of experiments.

CXCR3 and PD-L1 were highly expressed in SGC7901 but lowly expressed in MKN74 GC cell lines (Fig. 2a). After treatment of SGC7901 and MKN74 cells with 100 ng/ml CXCL9/10/11 for 72 h, the expression of PD-L1 was upregulated (Fig. 2b). After treatment of SGC7901 cells with 100 ng/ml CXCL9/10/11 for 5 min, 1 h, 4 h and 8 h, respectively, STAT3 and Akt were significantly activated, while ERK was not activated (Fig. 3a).

To further explore whether CXCR3 is responsible for the upregulation of PD-L1 and the activation of STAT3 and Akt in SGC7901 cells, we blocked CXCR3 with a specific inhibitor AMG487. Treatment with 1 μ M AMG487 prior to CXCL9/10/11 exposure diminished the upregulation of PD-L1, and reversed the activation of STAT3 and Akt induced by CXCL9/10/11 (Fig. 2c, 3b).

To investigate whether STAT3 and Akt are responsible for the upregulation of PD-L1 in SGC7901 cells, we blocked STAT3 and PI3K/Akt signaling with a STAT3-

specific inhibitor STAT3IC, a PI3K-specific inhibitor LY294002 and an Akt siRNA. When STAT3 and Akt activity were suppressed, treatment with CXCL11 diminished the upregulation of PD-L1 (Fig. 4), suggesting that CXCL11-CXCR3 upregulated PD-L1 probably by activating STAT3 and PI3K-Akt signaling pathways in GC cells.

We performed immunohistochemistry among 92 specimens of gastric cancer tissue, and we observed that the immune response of PD-L1 was mainly in the cytomembrane of gastric cancer cells, and the immune response of CXCR3 was mainly in the cytoplasm of gastric cancer cells (Fig. 2a). Among all the factors included, the expression of PD-L1 was correlated to TNM stage ($P = 0.018$), especially to I + II stage patients, but not correlated to gender, age, Lauren classification and lymph node metastasis of patients with gastric cancer. The expression of CXCR3 was correlated to gender ($P = 0.025$), especially to women patients, but not correlated to age, Lauren classification, TNM stage and lymph node metastasis (Table 1).

The expression of PD-L1 and CXCR3 was positively correlated in gastric cancer with a correlation coefficient of 0.211 ($P = 0.044$), with statistical significance (Table 2).

Discussion

PD-L1 expression is regulated by intrinsic and extrinsic pathways, and could be induced by hypoxia, toll-like receptor (TLR), gene mutations and cytokines. IFN- γ of the cytokine family is a key factor triggering PD-L1 induction in tumor cells. However, the specific mechanisms underlying PD-L1 expression remain largely unknown. Stimulation with IFN- γ , TLR ligands and LPS via MyD88, TRAF6, MEK, STAT1, NF- κ B and PI3K-dependent pathways increased PD-L1 expression in different types of cells, such as inflammatory cells, fibroblasts and cancer cells [27–30]. Additionally, IL-12, IL-27, IL-17 and TNF- α regulated PD-L1 expression in inflammatory cells and

Table 1 Relationship between the expression of PD-L1 and CXCR3 and clinico-pathological characteristics of gastric cancer patients

Clinico-pathological characteristics	Number	CXCR3 expression			PD-L1 expression			
		Low(%)	High(%)	<i>P</i> value	Low(%)	High(%)	<i>P</i> value	
Age(years)	<60	42	31(73.8)	11(26.2)	0.343	20(47.6)	22(52.4)	0.675
	\geq 60	50	41(82)	9 (18)		26(52)	24(48)	
Sex	Male	72	60(83.3)	12(16.7)	0.025	38(52.8)	34(47.2)	0.312
	Female	20	12(60)	8 (40)		8 (40)	12(60)	
pTNM stage	I + II	24	18(75)	6 (25)	0.652	7 (29.2)	17(70.8)	0.018
	III + IV	68	54(79.4)	14(20.6)		39(57.4)	29(42.6)	
N stage	Negative	23	17(73.9)	6 (26.1)	0.559	10(43.5)	13(56.5)	0.47
	Positive	69	55(79.7)	14(20.3)		36(52.2)	33(47.8)	
Lauren classification	Intestinal	41	31(75.6)	10(24.4)	0.372	21(51.2)	20(48.8)	0.941
	Diffuse	44	34(77.3)	10(22.7)		21(47.7)	23(52.3)	
	Mix	7	7 (100)	0 (0)		4 (57.1)	3 (42.9)	

Values shown in bold are statistically significant

Table 2 The correlation analysis of PD-L1 and CXCR3 expression in gastric cancer patients

PD-L1 expression	Number (%)	CXCR3 expression		r value	P value
		Low (%)	High (%)		
Low (%)	46(50)	40(87)	6(13)	0.211	0.044*
High (%)	46(50)	32(69.6)	14(30.4)		
Number (%)	92	72(78.3)	20(21.7)		

cancer cells by altering NF- κ B, STAT1 and ERK1/2 signaling [18, 19, 31]. However, our study showed that PD-L1 expression was enhanced via chemokine subfamily CXCL9/10/11-CXCR3 in a STAT3 and Akt-dependent manner in GC cells, suggesting that cytokines may induce PD-L1 expression through different STAT pathways. Our data do not support a role for ERK in inducing PD-L1 expression, but it seems likely that IFN- γ signaling is mediated through different pathways depending on the cell types involved.

CXCL9/MIG, CXCL10/IP10 and CXCL11/ITAC are members of the ELR-negative CXC chemokine subfamily, also known as IFN- γ -induced monocyte cytokines, IFN- γ -inducible protein 10 and IFN- γ -inducible T cell α chemokine [32–34]. CXCR3, the co-receptor of CXCL9/10/11, belongs to the seven-transmembrane G protein-coupled receptors (GPCRs) and is mainly expressed on activated T cells, NK cells, mast cells and dendritic cells. CXCR3 is reported to play a dual role in immunity and cancer. The CXCL9/10/11-CXCR3 signaling pathway in tumor micro-environment mainly facilitated the chemotactic movement of CXCR3 activated immune cells to the tumor site for anti-tumor immunity [35]. While CXCR3 expression was also detected in many cancers, such as breast cancer, malignant melanoma, renal cancer and colon cancer [36–39]. Some studies suggested that IFN- γ induced ELR-negative CXC chemokine expression, which can activate downstream MAPKs, PI3K-Akt and STAT by binding to their receptor CXCR3, and thereby promote tumor progression and metastasis. Meanwhile, IFN- γ as an immune factor plays an important role in the regulation of PD-L1. Interestingly, our study found that CXCR3 and PD-L1 were expressed in GC cells and tissues, and CXCL9/10/11-CXCR3 upregulated PD-L1 expression by activating STAT and PI3K-Akt pathways in GC through both data analysis and in vitro experiments, which meant that CXCR3 could play anti-tumor and pro-tumor roles in different types of cells.

Conclusions

In summary, our findings suggest a regulatory mechanism of PD-L1 through data analysis, in vitro and *vivo* experiments, which is an important factor of immune evasion in GC cells, and CXCL9/10/11-CXCR3 could regulate PD-L1 expression through STAT and PI3K-Akt

signaling pathways in GC cells. Recently, molecular targeted therapy has created hope for advanced GC patients. Checkpoint blockers such as PD-L1 offer novel immunotherapy options for cancer patients. Based on our findings, CXCR3 could be a potential target in GC therapy. Further studies are required to confirm this conjecture.

Additional files

Additional file 1: Table S1. The first 20 significantly enriched gene pathways in TCGA dataset. (XLS 18 kb)

Additional file 2: Table S2. The first 23 significantly enriched gene pathways in GSE15459 dataset. (XLS 21 kb)

Additional file 3: Table S3. The first 24 pathways significantly enriched gene pathways in GSE62254 dataset. (XLS 19 kb)

Additional file 4: Table S4. 81 genes that had a close relationship to PD-L1 were selected for Gene functional classification after spearman correlation analysis. (XLS 25 kb)

Additional file 5: Table S5. CXCL9/10/11 and CCL4 were grouped together after Gene functional classification in DAVID. (XLS 20 kb)

Abbreviations

CTLA-4: Cytotoxic T-lymphocyte-associated antigen; DAVID: The database for annotation, visualization and integrated discovery; FDR: False discovery rates; GC: Gastric cancer; GO: Gene ontology; GPCRs: Seven-transmembrane G protein-coupled receptors; IFN- γ : Interferon- γ ; IL-17: Interleukin-17; PD-1: Programmed death-1; PD-L1: Programmed death-ligand 1; TCGA: The Cancer Genome Atlas; TLR: Toll-like receptor; TNF- α : Tumor necrosis factor- α

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XQ and YL conceived and designed the study. CZ, TW and XW performed the experiments. LX and XC analyzed the data and prepared the manuscript. YF, CL and SW contributed the experimental reagents and materials. ZL and YC did the pathological diagnosis. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Human Ethics Review Committee of China Medical University; informed consent was obtained from all patients. The catalogue numbers for SGC7901 and MKN74 cell lines are TCHu 46 and JCRB0255.

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Department of Medical Oncology, the First Hospital of China Medical University, NO.155, North Nanjing Street, Heping District, Shenyang 110001, China. ²Department of Geratology, the First Hospital of China Medical University, Shenyang, Liaoning Province, China.

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