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Research article

STAT3 down-regulation for IL-2 inducing TIL-A clinical clue for spatial quantitative pathway analysis

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Abstract: Tumor-infiltrating lymphocytes (TILs) can be used in two clinical areas: Adoptive cell therapy (ACT) and studying TIL immune properties *ex vivo*. After we studied ACT for more than thirty years, we are now going to study T-cells' immune properties for personalized immunotherapy. A spatial-quantitative network model can be applied to analyze the immune properties of T-cells and then develop new strategies under a machine learning algorithm. This study utilized STAT3 complex down-regulation for IL-2 inducing cytotoxic T-lymphocyte (CTL) to study T-cell characteristics. After studying spatial-order regulation from the SMAD-STAT3 axis of multiple protein complexes, we focused on a second protein, or STAT3, knockdown for IL-2-inducing CTL. Because machine learning algorithms require a good pathway configuration to mine targeting genes and targeting drugs, the spatial-time quantification network can address the spatial competition of complex proteins binding to downstream proteins/DNA in network analysis. The research demonstrated that STAT3

downregulation can reactivate CTL under a spatial-order quantification network combined with IL2 inducing. After studying the algorithm of a spatial quantitative network under STAT3 downregulation, we can use the spatial-complex configurations to analyze complex regulation in artificial intelligence for future personalized therapy.

Keywords: STAT3 (Signal transducer and activator of transcription-3); tumor-infiltrating lymphocytes (TILs); quantitative pathway and network; spatial-timely quantitative pathway and network; cytotoxic T-lymphocyte (CTL); personalized therapy

1. Introduction

Tumor-infiltrating lymphocytes (TILs) are heterogeneous immune cells that kills tumor cells in clinical applications [1–7], but the cytotoxic T-lymphocyte (CTL) can be blocked by the immunosuppressive tumor microenvironment (TME) within tumors from myeloid-derived suppressor cells (MDSCs), regulatory T-cells (Tregs), and Programmed Death-Ligan 1 (PD-L1) [8–12]. Signal transducer and activator of transcription-3 (STAT3) is produced from MDSC and Tregs to participate in the TGF-β pathway, resulting in immune suppressive action in the TME [13]. STAT3 upregulation improves the propagation of tumor cells and crosstalk among vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 23 (IL-23) to promote tumor growth, angiogenesis, and invasion [14–17]. STAT3 can also inhibit the expression of immune-stimulatory factors, resulting in a TME decreasing immune responses to tumors [18,19]. Because we have reported that a spatial-timely quantification network can address the spatial competition of complex proteins binding to downstream proteins/DNA in network analysis which have been reported, we apply for the algorithm to study STAT3 downregulation. After we set up Suppressor of Mothers Against Decapentaplegic (SMAD)-STAT3 complex spatial-order regulation to compete for downstream binding sites, we use siRNA knockdown STAT3 to study the hypothesis due to SMAD-STAT3 complex inhibiting CTL functions. We used SMAD-STAT3 complex configuration under STAT3 downregulation to study IL-2-inducing CTL. The research goal is to confirm that spatial-order quantification network is more powerful than traditional pathway and study that STAT3 down-regulation will increase TIL activity. After studying SMAD-STAT3 complex downregulation in the spatial order quantitative network, we can use the spatial-complex configurations to analyze complex regulation in machine learning algorithms for future personalized therapy.

2. Materials and methods

2.1. Mining binding promoters of CTL genes

According to TIL CD8 immune networks which we have studied, at least three pathways are majorly involved in T-cell immune response to tumor cells: $CD8^+$ T-cell receptor (TCR) signal from TCR antigen associated with MHC class I; T-cells reactivated with IL-2 to produce Tumor necrosis factor alpha (TNF-α), Interferon-gamma (IFN-γ), Granzyme-B (GZMB), and perforin-1 (PRF1) to kill tumor cells; TGF-β pathway with SMAD-STAT3 combination to maintain quiescent TIL to block the antitumor functions in tumor tissues [20–22]. To study spatial-timely pathway and SMAD-STAT3

complex inhibiting CTL, we put promoters of IL-2, TNF-α, IFN-γ, GZMB, and PRF1 into TRANSFAC to screen binding protein (http://bioinfo.wilmer.jhu.edu/PDI/index.html) by using TRANSFAC Matrices (Cutoff < 0.001).

2.2. ChIP assay for STAT3-CTL gene promoter binding

After the discovery of STAT3 protein-predicted binding site in the promoters of IL-2, TNF-α, IFN-γ, GZMB, and PRF1, suggesting the STAT3 regulates CTL functions, two of the genes (TNF-α and IFN-γ) were studied by chromatin immunoprecipitation (ChIP). In detail, we used the cloned and cultured TIL from liver tumor tissues without IL-2 on day-7 and with IL-2 induction on day-10 for IL-2 induction 3 days as per our previous report and design [23–27]. The ChIP assay for the two groups was performed as routine protocol [28]. Briefly, both groups of cells were incubated with 1 mL lysis buffer (20 mM HEPES [pH 7.9], 350 mM NaCl, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF) on ice for 30 min, DNA fragmentation by sonication into 400–800 bp, Protein A (Thermo Scientific™ 88802) beads with 1 μg STAT3 antibodies were incubated for two hours under at 4 ℃ to pull down the bound DNA fragments. The fragments with the appropriate size of DNA were purified and ran PCR. AP1 also binds the promoters of TNF-α and IFN-γ, so AP1 was used as a positive control and IgG was used as a negative control.

2.3. STAT3 down-regulation for CTL gene expression

After discovering the SMAD-STAT3 DNA binding site within promoters of TNF-α, IFN-γ, GZMB, and PRF1, we used siRNA STAT3 to downregulate STAT3 and study inhibited STAT3 regarding enhancing CTL genes' expression. The culture cloned TIL from liver tumor tissues was cultured for 21 days as the previous report [23–27,29,30]. In detail, after culturing from day-0 to day-12, the growth medium was replaced with Opti-MEM Invitrogen, Carlsbad CA). For each transfection, 100 nm and 200 nm targeting STAT3 siRNA (CAT#: SR321907, Origene) with scrambled control using DharmaFECT (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. Each experimental condition was performed in triplicate. After cells were transfected for 72 hours, the cells were harvested and assayed for mRNA and protein for CTL gene expression, as in our previous reports [31–33]. Briefly, mRNA expression was measured for STAT3 downregulation with two of the CTL genes, TNF-α and INF-γ, by Q-rtPCR. Western blot is used as per our previous manuals [34–36] to confirm the protein level of TNF-α and INF-γ with STAT3 down-expression (STAT3 antibodies are ab31370).

2.4. Experimental support STAT3-CTL regulation

To study CTL from siRNA STAT3 down-regulation, CTL function was defined by MTT as previous manual reports [37–40]. Briefly, CTL was assayed for the HepG2 cell line as our report [41,42]. The cells were seeded in 96-well tissue culture plates at a density of 5×10^3 cells/well. TIL was measured for effector-to-target ratio by 1:25 and 1:50 at culture from day-15. On the day-15, the medium was removed and 100 μL of fresh medium containing 0.5 mg/mL MTT (Roche; Manheim, Germany) was added to each well. The cells were incubated at 37 ℃ in a humidified 5% CO_2 atmosphere for 4 hours, followed by the addition of 150 μL of solubilization solution (0.01 mol/L HCl in 100 g/L sodium dodecyl [SDS]) to each well and the incubation of cells for a further 10 minutes at 37 ℃ with gentle

shaking. The optical density of the plates was measured using spectrophotometric absorbance at 570 nm in the Microplate Reader Model 550 (Bio-Rad; CA).

2.5. In silico support quantitative network under Smad-STAT3 complex

According to the research purpose, the final mimic included IL2 activating pathway with CTL under TNF-α, INF-γ, GZMB, and PRF1 participation and blocked STAT3 within the network of TCR signal and IL2 activating pathway. To study the spatial-timely quantitative network from the SMAD-STAT3 complex regulation, TCR signal/and IL2 activating pathway/quiescent TIL-cell pathway hubs derive from our previous published genomic profile [26,43,44] and targeting SMAD-STAT3 within TCR signal and IL2 activating pathway from published STKE database. The complex regulation within spatial-timely quantitative network modeling is SMAD-STAT3 complex (Smad-2, Smad-3, and Smad-4) with combination ratio (0%, 25%, 50%, 75%, and 100%) and downstream transcriptional factor complex binding sites with combination ratio (0%, 25%, 50%, 75%, and 100%) under our Python script plugin into Cytoscape (http://www.cytoscape.org/) which we have written and reported [45,46]. We are finally combining targeting SMAD-STAT3 with IL2 activating pathway for CTL under blocked STAT3 status.

3. Results

3.1. Mining binding promoters of CTL genes under STAT3 transcriptional factors with confirmation

According to TIL CD8 immune networks, T-cells reactivated with IL-2 to produce TNF-α, IFN-γ, GZMB, and PRF1 to kill tumor cells. In order to discover STAT3 inhibiting CTL functions and to study spatial-timely pathways, we put promoters of IL2, TNF-α, IFN-γ, GZMB, and PRF1 into a platform of transcriptional factors binding screening (https://biogrid-lasagna.engr.uconn.edu/lasagna_search/). As Figure 1 shows, there are two matrix logos as STAT3 protein binding promoters: M00225 (Figure 1A) and M00497 (Figure 1B). As Table 1 shows, STAT3 protein in silico binding sites are demonstrated within the promoter of TNF-α, IFN-γ, GZMB, PRF1, and IL2, in which STAT3 has five binding sequences in PRF1 promoter, four binding sites within that of IL2, three binding sites within that of TNF-α and two binding sites within that of IFN-γ and GZMB. As Table 1 shows, the P-values are satisfactory within both strands.

Because the promoters of CTL genes were discovered for binding by STAT3 proteins, two of the genes (TNF-α and IFN-γ) are applied for chromatin immunoprecipitation (ChIP) assay. After we harvested the cultured cloned TIL from liver tumor tissues without IL-2 groups on day-7 and on day-10 after IL-2 induction, we performed a chromatin immunoprecipitation (ChIP) assay. We observed low TNF-α and IFN-γ binding on day-7 and high TNF-α and IFN-γ binding about 2.5-times significantly increased on day-10 ($P = 0.044$ and $P = 0.0072$, respectively). The results showed that STAT3 regulates TNF-α and IFN-γ expression after IL2 induction TILs. Because AP1 located with TNF-α and IFN-γ promoter was predicted in silico, AP1 was used as a positive control, as shown in Figure 1C, while IgG was used as a negative control.

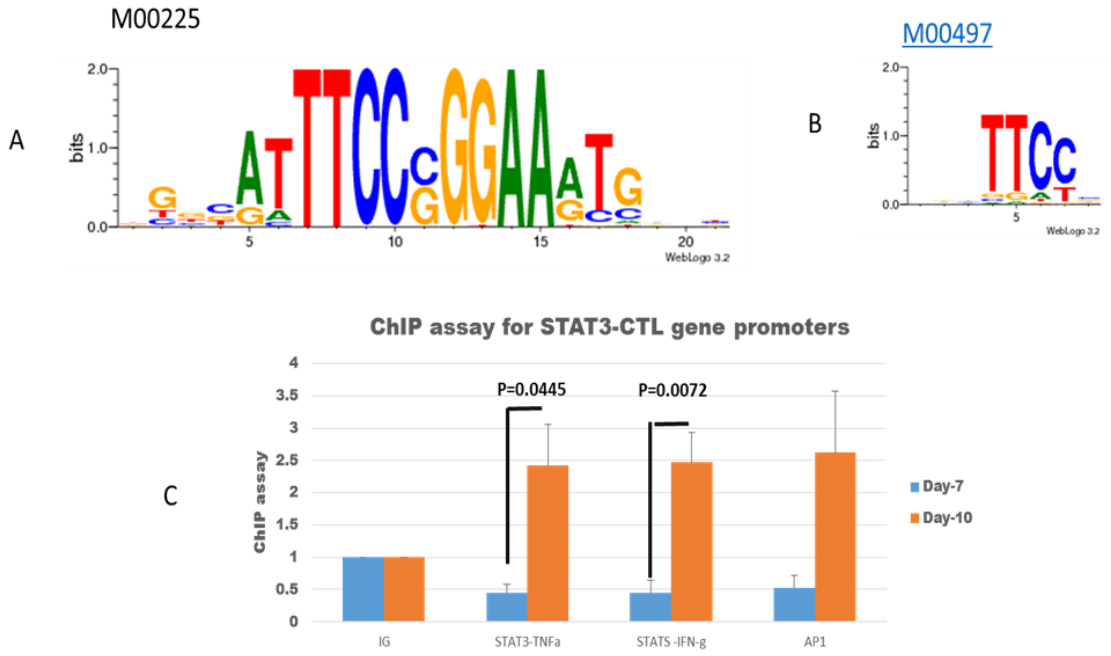

Figure 1. STAT3 promoter binding Logo and ChIP assay. A) Shown M00225 logo; B) for M0049 logo; C) Observed low TNF-α and IFN-γ binding at day-7 and compared to about 2.5-times significantly increase by day-10, respectively. Because AP1 also located with TNF-α and IFN-γ promoter was predicted by in silico, AP1 was used as a positive control as shown, while IgG was used as a negative control as shown.

Table 1. CTL gene promoter binding motif.

Name of promoters	Binding protein name	Promoters sequence	Position	Strand	Score	P-value	E-value
TNF-alpha	STAT3 (M00225)	CCGGGAATTCACA	608	−	12.52	0.0002	0.192
TNF-alpha	STAT3 (M00497)	AATTCCC	612	+	7.3	0.00065	0.63
TNF-alpha	STAT3 (M00497)	GAATTCCC	611	+	8.33	7.50E-05	0.073
INF-gamma	STAT3 (M00225)	CGGAAACGATG	466	+	11.26	0.0004	0.38
INF-gamma	STAT3 (M00497)	GAGTTCCC	868	−	7.58	0.0003	0.292
GZMB	STAT3 (M00225)	CGGAAATGCCA	642	−	12.81	0.000125	0.146
GZMB	STAT3 (M00497)	GAGTTCCC	809	−	7.58	0.0003	0.3
PRF1	STAT3 (M00225)	CGGGAAAAGAGA	132	−	10.47	0.00075	0.93
PRF1	STAT3 (M00225)	GGAAGTGGAT	794	+	11.54	0.0004	0.5
PRF1	STAT3 (M00497)	AATTCCA	1	+	7.31	0.00045	0.56
PRF1	STAT3 (M00497)	GATTCCG	318	+	7.79	0.000175	0.219
PRF1	STAT3 (M00497)	AGATTCCG	317	+	8.29	0.000125	0.157
IL2	STAT3 (M00497)	GAATTCCA	0	+	8.35	5.00E-05	0.063
IL2	STAT3 (M00497)	AGATTCCG	317	+	8.29	0.000125	0.157
IL2	STAT3 (M00497)	GATTCCG	318	+	7.79	0.000175	0.219
IL2	STAT3 (M00497)	AATTCCA	1	+	7.31	0.00045	0.56

3.2. Results of STAT3-knockdown with CTL gene expression and CTL function

Two of the three pathways are majorly involved in T-cell immune CTL to kill tumor cells: $CD8^+$ TCR signal from TCR antigen associated with MHC class I and T-cells reactivated with IL-2 to induce TNF-α, INF-γ, GZMB, and PRF1.

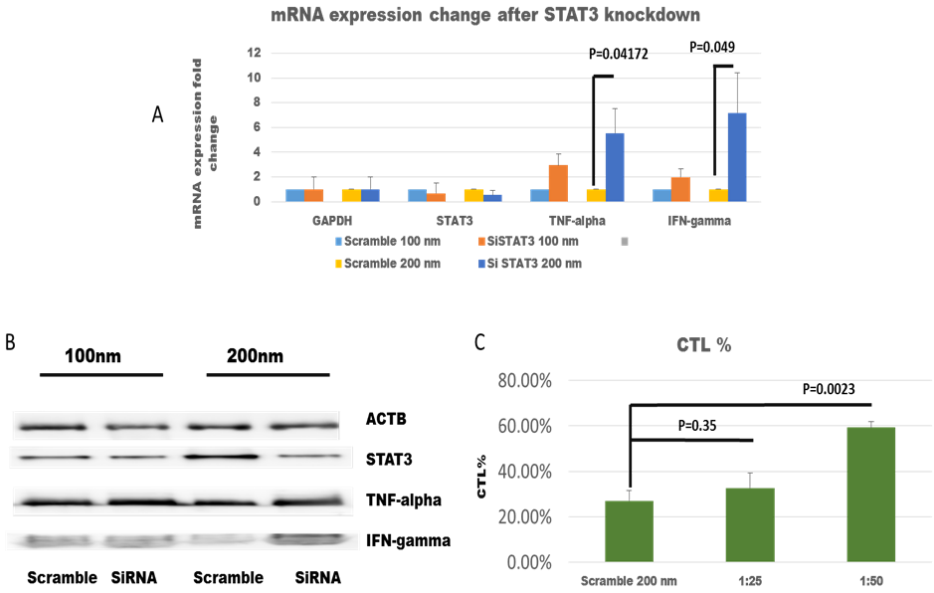

Figure 2. STAT3 downregulation for CTL gene expression and function change. A) Day-15 RT-qPCR quantification of targeting STAT3 mRNA. B) Confirmation of STAT3 downregulation by Western blot at day-15. C) Confirmation of STAT3 downregulation by CTL increase at day-15.

After STAT3 was mined by transcriptional binding in promoters of TNF-α, INF-γ, GZMB, and PRF1 for CTL, according to the research goal, two of the CTL genes (TNF-α and IFN-γ) were also studied for the gene expression after STAT3 knockdown [23,24,47,48]. The results showed that STAT3 down-regulating will increase TNF-α and IFN-γ expression as Figure 2A indicates that down-regulation STAT3 is expressing a level of 50% in the 100 nm group and 37% in the 200 nm group. TNF-α mRNA is 2.54 and 5.54 times higher transfected TILs than those in the scramble control group to compare 100 nm and 200 nm scramble TIL group (significantly increased in 200 nm group, P = 0.04172), respectively. The IFN-γ level is about 1.99 and 7.18 times higher in the transfected TILs group than those in the 100 nm and 200 nm scramble group (significantly increased in the 200 nm group, P = 0.049). As Figure 2B, Western blot further confirmed TNF-α and IFN-γ higher expression after STAT3 down-regulation.

After STAT3 was downregulated to confirm the increase of TNF-α and INF-γ to expression, the culture cloned TIL was further studied CTL function assay under TIL for effector: target ratio by 1:25 and 1:50 at culture from day-12 to day-15 as our previous report [25,42,49,50]. The results of CTL killing activity showed about 31% (1:25) and 59% (1:50) at 200 nm siRNA STAT3 in the transfected TILs group to compare the scrambled group (about 25%) at 200 nm shown in Figure 2C. Obviously, cytotoxic T lymphocyte of knockdown STAT3 TIL was (significantly increased on 1:50, P = 0.0023) much higher than scrambled groups.

3.3. In silico results for mimic complex for spatial-timely quantitative network

To study spatial-timely quantitative network from the complex proteins and STAT3 downregulation for reactivating IL-2 induced TILs, in silico experiment is SMAD-STAT3 complex regulation from TGF-β/SMAD signaling for TCR signaling and IL-2 reactivated to kill tumor cells. After the spatial-timely quantitative network set up with SMAD combination-regulation ratio (0%, 25%, 50%, 75%, and 100%), the results demonstrated SMAD-STAT3 complex regulation ratio (0%, 25%, 50%, 75%, and 100%). Eventually, we can harvest maximal spatial-timely combination-regulation with the activity of SMAD-STAT3 complex including 15,625 combination regulation as Table S1, including maximal reactivity values (spatial-timely order) for SMAD-STAT3 complex, so that results in silico can discover maximal regulation from a TGF-β/SMAD-STAT3 to regulating CTL functions. IL-2-inducing CTL genes are shown in Figure 3A. Additionally, we can achieve combination regulation activity for SMAD-STAT3 complex regulation, as shown in Figure 3B. The results demonstrated that IL-2 is completely blocked under STAT3 higher expression as Figure 3C2. Further, during in silico experiments, as demonstrated in Figure 3D, STAT3 is blocked to 70% CTL function to increase relating with IL2 inducing will show liner increase under the maximal spatial-timely combination-regulation Figure 3D2.

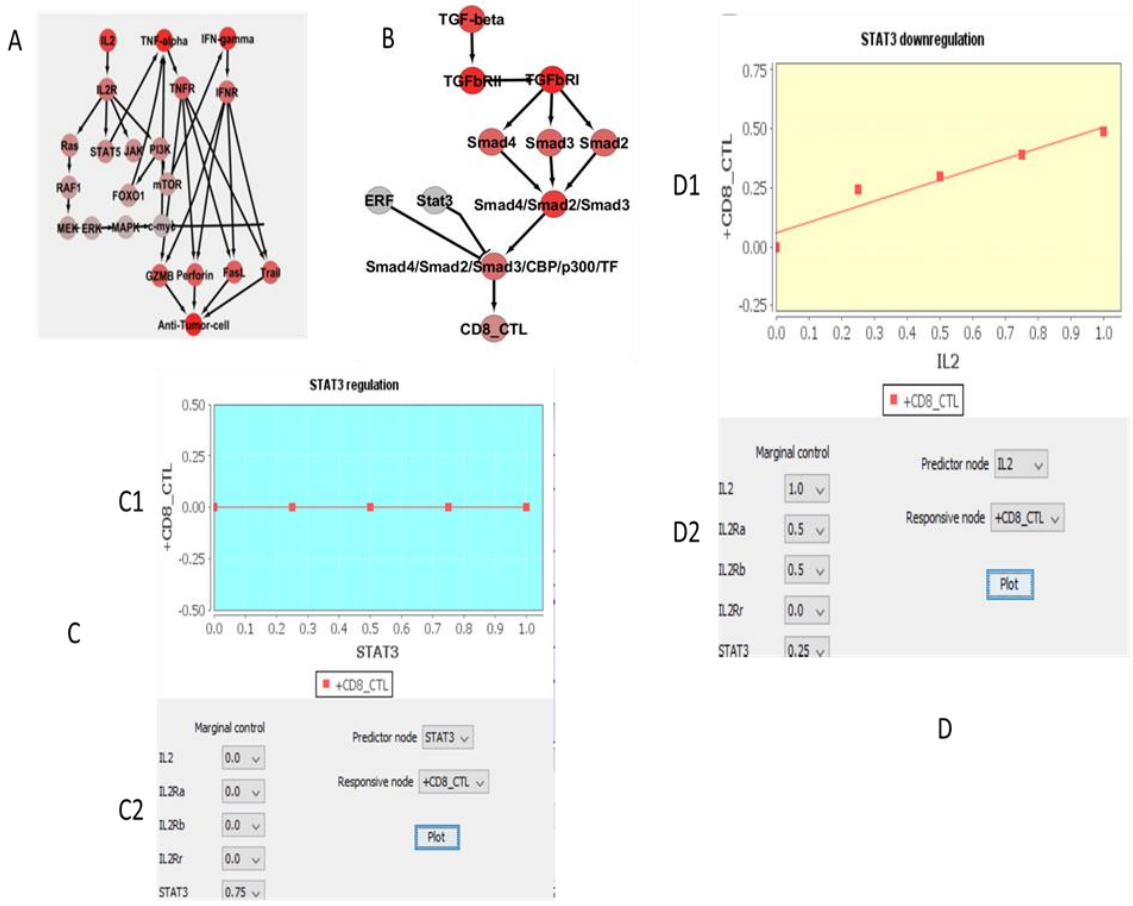

Figure 3. In silico support Smad-STAT3 complex to CTL. A) Shown is a CTL pathway; B) STAT3 in CTL pathways; C) Smad-STAT3 complex regulation, as Figure 3C2; D) STAT3 is blocked, CTL function to positively related with IL2 inducing IL2 inducing will show liner increase under the maximal spatial-timely combination-regulation. Figure 3D2.

4. Discussion

Tumor-infiltrating lymphocytes are a group of heterogeneous T-cells that have relevance in administrating TIL adoptive cell therapy (ACT) [51] and studying immune characteristics regarding T-cell, B-cell, NK cells, macrophage, and neutrophil within the tumor tissues [5,7,52,53]. In order to study efficacy in treating solid tumor diseases, we began to study TIL genomic profiles from single-cell mRNA differential display from TIL published in 2007 [31] to single-cell RNA-seq from TIL published in 2013 [54], establishing a TIL quantitative network published in 2015 [55] and studying machine learning algorithms by artificial intelligence (AI) from TIL published in 2022 [56].

Because the SMAD-STAT3 complex is focused on immune tolerance and T-cell quiescence through multiple mechanisms, as an oncogene, STAT3 was first described by Takeda et al. in 1999 as a negative regulator of Th1 immune responses [57], and other studies further support the role of STAT3 in immune evasion of human cancers [58]. The evidence demonstrated that multiple oncogenic signaling pathways regulate STAT3 expression in cancer cells, one of those from JAK/STAT3 [59] while the SMAD-STAT3 axis controls immune effects in the TGF-β pathway [60]. The TGF-β pathway has two effects same "double-edged sword" as "immune stimulation and inhibition". Here, we report that STAT3 signaling attenuates immune responses through direct SMAD3-STAT3 interaction. Activated STAT3 suppresses the immune response with SMAD3, causing STAT3 and SMAD3 to block some immune responses. The evidence demonstrated that STAT3 activation suppresses the expression of CTL functions, such as IFN-γ, which are required for innate and adaptive antitumor immunity [61]. These findings suggest that STAT3 blockade could be used in cancer immunotherapy with increasing CTL functions.

According to these investigations, the spatial-timely quantitative network can mimicking complexes binding to complexes regulation because three pathways are majorly involved in T-cell immune CTL to tumor cells: $CD8^+$ TCR signal from TCR antigen associated with MHC class I; T-cells reactivated with IL-2 to induce TNF-α, INF-γ, GZMB, and PRF1 and TGF-β pathway for quiescent TIL-cell in tumor tissues. After we found that STAT3 has a binding site in promoters in CTL genes such as TNF-α, INF-γ, GZMB, and PRF1 while SMAD-STAT3 complex also involves the four promoters related to TGF-β/SMAD signaling-dependent activation of the TGF-β pathway, STAT3 down-regulation can be used to study CTL function and spatial-timely pathway. STAT3 knockdown results demonstrated TNF-α and IFN-γ higher expression than our previous results from traditional pathway configuration [62−65]. Moreover, Western blot not only supported mRNA results but also CTL increase after knockdown STAT3. The experiment data and in silico data support the spatial-timely quantitative pathway related to the TIL function. Now we can use the algorism to activate and inhibit the genes by targeting in vivo for combination therapy or *ex vivo* for analysis of other immune cells [66].

5. Conclusion

CTL can be blocked by the immunosuppressive TME within tumors. STAT3 is key to the TGF-β pathway, resulting in immune suppressive action. In order to study STAT3 regulation, we study the SMAD-STAT3 complex within a spatial-order regulation pathway. Therefore, we use siRNA knockdown STAT3 to study the SMAD-STAT3 complex regulating CTL functions. We discovered the complex configuration of SMAD-STAT3 complex downregulation that induces CTL functions.

The research evidence supported spatial-order quantification networks with more power than traditional pathways and confirmed that STAT3 down-regulation increases TIL activity. After studying the SMAD-STAT3 complex downregulation in the spatial order quantitative network, we can use the spatial-complex regulation algorithm to perform a machine learning algorithm for "big-data" analysis for personalized therapy.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

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Conflict of interest

Biaoru Li is an editorial board member for AIMS Allergy and Immunology and was not involved in the editorial review or the decision to publish this article. All authors declare that there are no competing interests.

Author contributions

YY (1) all papers with references check, VX for ChIP with statistics support and SYC rtPCR and CTL with statistics support. WQL and JD perform data, bioinformatics and biostatistics, YY (5) teaches YY (1) rtPCR and Western blot and VX performs rtPCR and Western blot under BL. BL conceived and designed the experiments.

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