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Original Paper

Plasminogen Activator Inhibitor 1 Promotes Immunosuppression in Human Non-Small Cell Lung Cancers by Enhancing TGF-B1 Expression in Macrophage

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Key Words
Hegdhr • NSCLC • TGF-β • PAI-1 • TAMs

Abstract
Background: Plasminogen activator inhibitor-1 (PAI-1) has been regarded as a risk factor for thrombosis and atherosclerosis. Since it has been shown that PAI-1 can activate macrophages through Toll-like receptor-4, we sought to investigate the role of PAI-1 in the tumor microenvironment. ***Methods:*** The expression and distribution patterns of PAI-1 and transforming growth factor beta (TGF-β) were measured in 60 non-small cell lung cancer (NSCLC) tumors. A statistical correlation analysis was performed between PAI-1 and TGF-β expression and distribution in each tumor. The distribution of tumor-associated macrophages (TAMs) was also measured and its correlation to PAI-1 levels was analyzed. Levels of secreted CCL-17, CCL-22, IL-6 and TGF-β were measured in cell cultures of human macrophage cell lines THP-1 and U937 treated with PAI-1. Levels of secreted PAI-1 were monitored in cell cultures of human NSCLCs cell lines 95D and A549 treated with TGF-β. Secreted proteins were measured in cell culture supernatants using ELISA. Changes in downstream signaling pathways were investigated using western blot. ***Results:*** PAI-1 and TGF-β were found to be overexpressed in human NSCLCs. PAI-1 expression was tightly correlated to TGF-β expression as well as the percentage of TAMs. PAI-1 treatment increased the expression of TAM-associated cytokines and chemokines, including CCL-17, CCL-22, and IL-6. PAI-1 treatment was also observed to enhance TGF-β expression in macrophage cell lines through an IL-6 autocrine/paracrine manner. The effects on TGF-β expression were blocked by NF-κB and STAT3 inhibition. Interestingly, TGF-β also increased levels of secreted PAI-1 in NSCLC cells through SMAD3-dependent signaling, therefore resulting in a feed-forward loop. However, this loop could be blocked by NF-κB, STAT3 and SMAD3 signaling inhibition, as well as treatment with a high concentration of TGF-β. ***Conclusion:*** PAI-1 and TGF-β promote NSCLC tumor cells and TAMs and might be valuable targets for cancer immunosuppression.

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Introduction

Immunosuppression refers to the attenuation of a healthy immune response toward antigens, either deliberately through administration of immunosuppressive drugs, or as an adverse effect of a therapeutic agent such as anti-neoplastic chemotherapy [1, 2]. Recently, anti-cancer immunity has been the focus of cancer research. In general, the degree of tumor immunosuppression is determined by several factors including the ability of tumor cells to generate an immune-tolerant microenvironment, the activation of negative regulatory immune checkpoints in the tumor microenvironment and the secretion of immunosuppressive cytokines and soluble inhibitory factors [1, 2]. The tumor microenvironment is dictated mainly by the interaction between tumor cells and the immune system. The interaction between these two groups of cells is mostly indirect, through the release of soluble cytokines and chemokines. Transforming growth factor beta (TGF-β) is one of the most essential negative immunomodulatory cytokines.

TGF-β is a multifunctional cytokine belonging to the transforming growth factor superfamily. Within tumors, TGF-β can be triggered by several factors including pH change, reactive oxygen species and thrombospondin-1. TGF-β can be secreted by cell types such as macrophages and T cells, which further acts on neighboring tumor and immune cells [3-6]. Besides the pro-apoptotic and pro-metastatic effects TGF-β has on tumor cells, its main effects are to induce immunosuppression by promoting Treg cell differentiation, inhibition of B cell proliferation, and inhibition of inflammation mainly by blocking the activation of NF-κB[7]. Recently, several studies have revealed that TGF-β also regulates thrombosis by promoting the expression of plasminogen activator inhibitor-1 (PAI-1) [8, 9].

PAI-1 is also known as endothelial plasminogen activator inhibitor, encoded by the *SERPINE1* gene. It is widely known that elevated PAI-1 is a risk factor for thrombosis and atherosclerosis. The primary function of PAI-1 is to inhibit urokinase plasminogen activator (uPA), which is an enzyme that cleaves plasminogen, thereby producing plasmin [10]. PAI-1 can inhibit uPA via active site binding, which subsequently prevents the formation of plasmin. The role of PAI-1 in cancer has been unclear. Recently, Gupta et al. reported that PAI-1 can activate macrophages through Toll-like receptor-4 (TLR4)[11]. Our preliminary data also indicated that the expression of PAI-1 was positively-correlated to levels of secreted TGF-β in human non-small cell lung cancer (NSCLC), which was measured by using immunohistochemistry (IHC) staining. Therefore, we sought to investigate the role of TGF-β and PAI-1 in the interaction between cancer cells and the immune system.

Materials and Methods

Patients

A total of 72 NSCLC cases and 91 sex- and age-matched controls were obtained from Jiangsu Province Hospital and were included in the study. Patients were consecutively recruited between February, 2012 and January, 2016. All cases are incident once during enrollment of the current case-control study. The diagnosis of all patients was pathologically confirmed. A face-to-face questionnaire was administered to all patients to collect demographic data. Written informed consents were obtained from all participants. This study was approved by the Institutional Review Board of Jiangsu Province Hospital.

Cell lines and reagents

Human monocyte cell lines U937 and THP-1 were purchased from the cell bank of China Science Academy and human NSCLC cell lines 95D and A549 were purchased from ATCC. The cells were maintained in a humidified 37°C incubator with 5% CO_2. For macrophage differentiation, U937 and THP-1 cells were treated with 100 ng/mL PMA (Sigma–Aldrich, St. Louis, MO, USA) for 2 days. Human recombinant PAI-1 and TGF-β were purchased from Thermo Fisher (RP-75686 for PAI-1 and 14-8348-62 for TGF-β) and used at the concentrations mentioned in the results. Inhibitors against NF-κB, STAT3 and SIS3 were purchased from Selleckchem (BAY 11-7082:S2913; BP-1-102:S7769; SIS3:S7959). A commercial siRNA kit targeting human

TLR4 was purchased from Santa Cruz (sc-40260-SH, CA) and the expression of TLR4 was knocked down per the manufacturer's instructions. For the interleukin (IL)-6 antibody blocking assay, the cells were treated with a final concentration of 50 μg/mL of the anti-IL-6 antibody (Santa Cruz) (sc-130326).

Immunohistochemistry staining

Tissue sections were deparaffinized and rehydrated with a graded ethanol series and distilled water, and then treated with 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. Tissue sections were then rinsed twice for five minutes in phosphate-buffered saline (PBS) and incubated with 10% normal goat serum for 30 min to block non-specific antibody binding. After washing, the samples were incubated with a primary anti-rabbit Abcam (Cambridge, MA, USA) antibody (PAI-1 (ab142349), CD163 (ab87099) and TGF-β (ab2486)) at 4°C overnight. Sections were then washed in PBS three times and incubated with secondary antibodies. The sections were then stained with DAB per the manufacturer's protocol, mounted on slides, and photographed using a digital microscope camera (Nikon, Tokyo, Japan).

Western blot

For western blotting, proteins were extracted from tissues or cultured cells using RIPA buffer containing phenylmethanesulfonylfluoride (PMSF) (Beyotime, Nantong, China). Equal amounts of protein (100 μg) were separated by SDS-PAGE on a 7.5%/12.5% gel and transferred to a PVDF membrane. Primary polyclonal antibodies targeting IκBα(ab7217), IκBα s36 (ab133462), IL-6 (ab6672), TGF-β (ab2486), PAI-1 (ab142349), SMAD3 (ab40584) and p-SMAD3 s425 (ab51177) were purchased from Abcam. The secondary antibodies used were HRP-conjugated anti-rabbit or anti-mouse antibodies and were purchased from Santa Cruz Biotechnology. The blots were developed using ECL reagent (Millipore, MA, USA). An equal amount of loaded protein in each lane was confirmed using a β-actin antibody. ImageJ software was used to quantify the integrated density of the band.

ELISA

Levels of PAI-1, TGF- β, CCL-17, IL-6 and CCL-22 were measured in the supernatants from cell cultures with a commerical ELISA kit (Thermo Fisher; BMS2033, 88-8350-86, EHCCL17, BMS213-2 and EMCCL22). The experiments were carried out according to the manufacturer's instructions.

3D co-culture system

Differentiated THP-1 and 95D cells were cultured in a 3D Petri Dish (Micro-Tissues) (RI, USA) per manufacturer's instructions. Cell culture supernatants were collected daily for seven days. The cells were then collected, washed with PBS, and stained.

Statistical analysis

Differences between cases and controls were evaluated using the Student's t-test for continuous variables and the $\chi 2$ test for categorical variables. A linear correlation analysis was performed and tested with the F-test. The overall survival of different groups were analyzed using a Kaplan-Meier curve. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS Version 12) software.

Results

PAI-1 expression was correlated to TGF-β expression in human NSCLC

Since PAI-1 expression was reported to increase in human NSCLC significantly [12], and PAI-1 can activate the macrophages through TLR4[11], we proposed that PAI-1 might be involved in immunosuppression. NSCLC tumors from 60 patients were stained for PAI-1 and TGF-β. Twenty-one tumors exhibited strong PAI-1 staining, 28 tumors exhibited medium/weak PAI-1 staining, and 11 tumors were PAI-1-negative (Fig. 1A and B). Images indicated that PAI-1 was mainly distributed within tumor cells as well as in the extracellular matrix. PAI-1 was also distributed within non-tumor cells, such as immune cells. For TGF-β staining, 19 tumors exhibited strong staining, 30 tumors had medium/weak staining, and 11 tumors were negative (Fig. 1A and C). The expression of both PAI-1 and TGF-β were

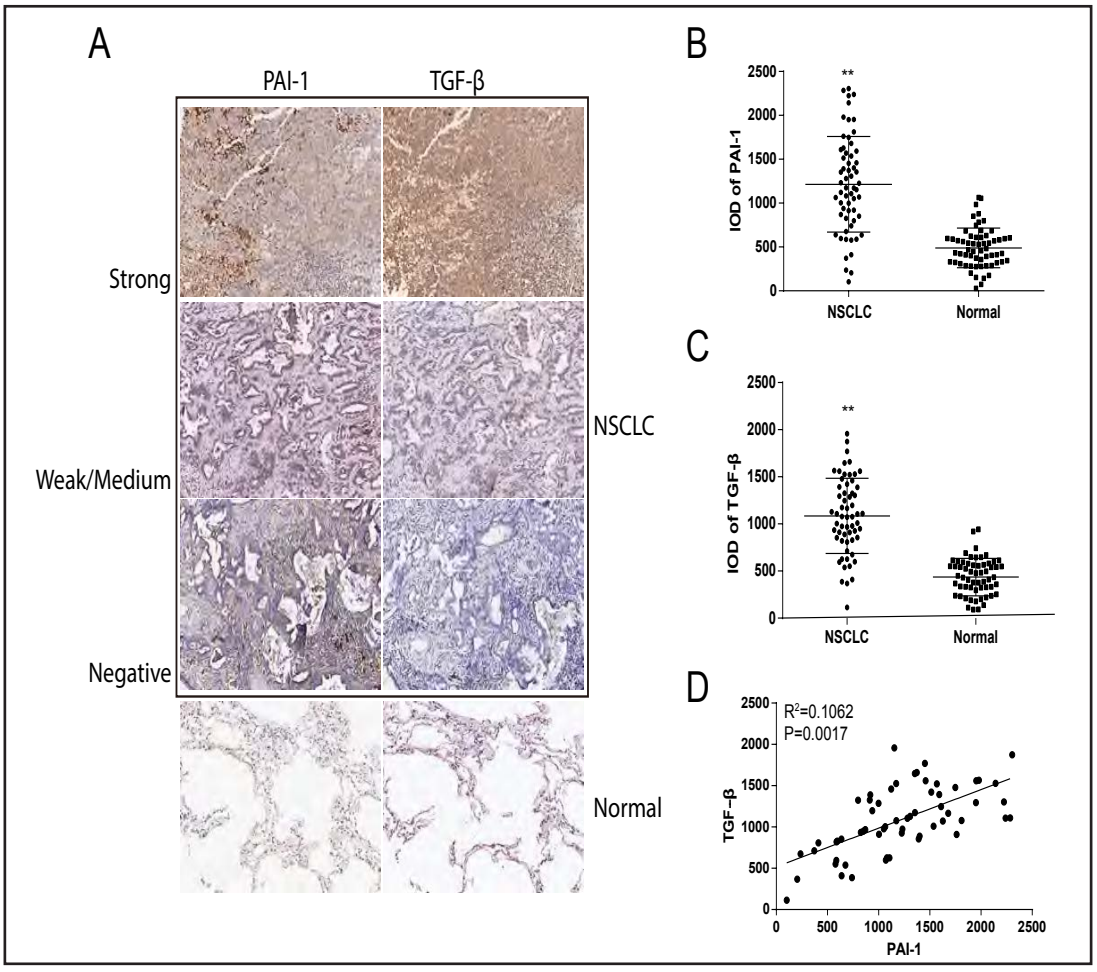

Fig. 1. PAI-1 expression correlates to TGF-β expression in human NSCLC. A) PAI-1 and TGF-β expression were measured with IHC staining. Representative images showing strong, weak/medium and negative staining in NSCLC and normal lung tissue for both proteins are displayed. B, C) Integrated Optical Index (IOD) values were quantified from PAI-1 and TGF-β IHC staining and are graphed. The IOD values of five random fields of view for each sample were measured using Image J software. D) A linear correlation analysis was performed between IOD values of TGF-β and PAI-1 in human NSCLC. Each experiment was performed in triplicate. Data are presented as the mean ± SEM. * indicates P<0.05 and ** indicates P<0.01.

significantly increased in NSCLC compared to adjacent normal lung tissues (Fig. 1A, B, and C). Furthermore, a linear correlation analysis was performed on PAI-1 and TGF-β IHC staining in the 60 tumors. The results indicate that the expression of PAI-1 is tightly correlated to the expression of TGF-β (R^2=0.4602, P=0.0017) (Fig. 1D).

PAI-1 expression was seriously associated with tumor associated macrophages (TAMs) distribution in human NSCLC

To investigate the relationship between TAM distribution and PAI-1 expression, we first divided the 60 NSCLC patients into two subgroups according PAI-1 expression. The upper 95% confidence interval of PAI-1 expression in healthy patients was used to stratify these patients into the two groups ($PAI\text{-}1^{High}$, n=24 and $PAI\text{-}1^{Low,}$ n=36). The distribution of TAMs was determined in both groups using the well-characterized marker CD163. The expression of CD163 was significantly higher in the $PAI\text{-}1^{High}$ group compared to the $PAI\text{-}1^{Low}$ group (Fig. 2A and B). This may indicate that PAI-1 expression is associated with TAM differentiation and function. To elucidate this, we determined serum concentrations of CLL-17 and CLL-

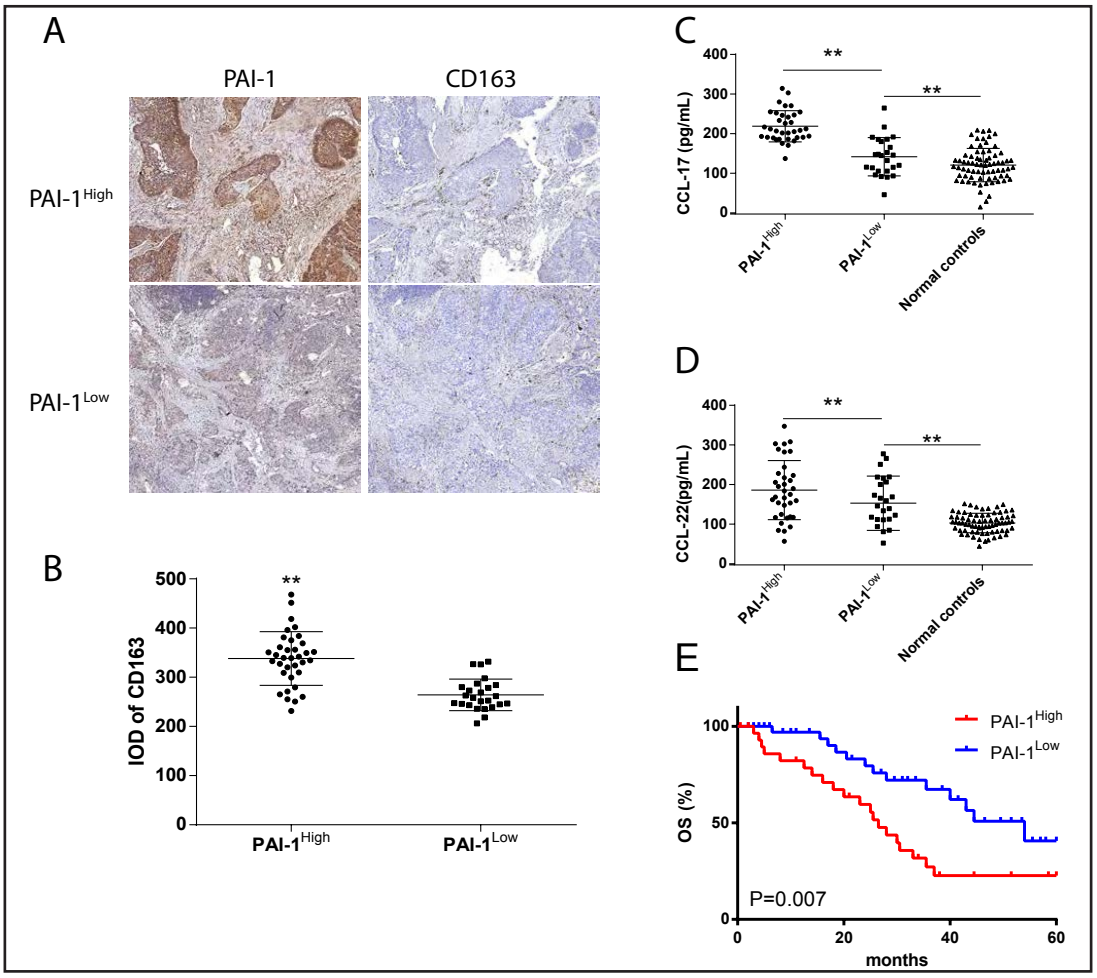

Fig. 2. PAI-1 expression is associated with increased tumor associated macrophage (TAM) distribution in human NSCLC. A) The expression of PAI-1 and CD163 were measured using IHC staining of the PAI-1^{High} and PAI-1^{Low} tissues. B) IOD values of CD163 within the PAI-1^{High} and PAI-1^{Low} groups were quantified and graphed. C, D) Serum CCL-17 and CCL-22 were measured using ELISA in the PAI-1^{High}, PAI-1^{Low} and normal controls. E) Overall survival rates (OS) of NSCLC patients post-surgery were analyzed with Kaplan–Meier survival curves. Each experiment was performed in triplicate. Data are presented as the mean ± SEM. * indicates P<0.05 and ** indicates P<0.01.

22, which are well-characterized TAM-associated chemokines. Significantly higher levels of CCL-17 and CCL-22 were found in NSCLC patients compared to healthy controls, and the concentration of both CCL-17 and CCL-22 were also significantly higher in the PAI-1^{High} group compared to the PAI-1^{Low} group (Fig. 2C and D). IHC staining indicated that PAI-1 is mostly expressed in tumor cells, indicating that there could be effects on TAM differentiation through TLR. The effect on TAMs is further explored later. We also performed a follow-up study to track survival of NSCLC patients post-surgery in the PAI-1^{High} and the PAI-1^{Low} groups. Patients in the PAI-1^{High} group had significantly shorter survival times (overall survival [OS]=26.5%) compared to patients in the PAI-1^{Low} group (OS=54%; P=0.007) (Fig. 2E). The results imply that PAI-1 might be involved in immunosuppression and could serve as an indicator of poor prognosis in NSCLC patients.

PAI-1 can promote TGF-β secretion in macrophage by increasing the production of IL-6

The effect of PAI-1 treatment on the secretion of cytokines by macrophages was investigated. Two human monocyte cell lines, U937 and THP-1, were differentiated to

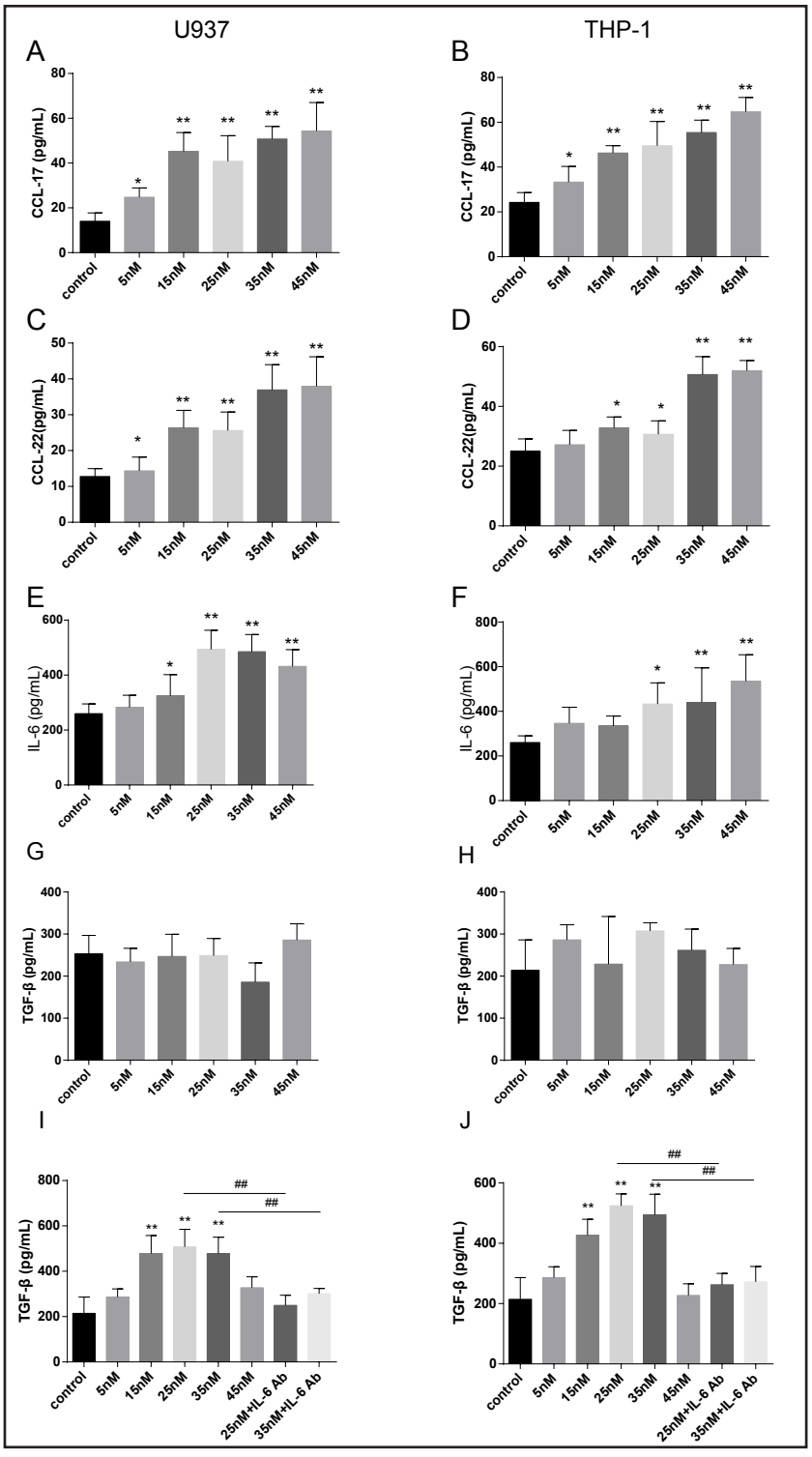

Fig. 3. PAI-1 promotes TGF-β secretion in macrophages by increasing IL-6 production. A, B) The concentration of CCL-17 was determined using ELISA in differentiated U937 and THP-1 macrophage cell culture media 48 h post-PAI-1 treatment of varying concentrations. C-H) Concentrations of CCL-22, IL-6 and TGF-β were determined by ELISA under the same experimental conditions as in A and B. I, J) The concentration of TGF-β was determined by ELISA in differentiated U937 and THP-1 macrophage cell culture media 72 h post-PAI-1 treatment of varying concentrations. Cultures were also treated with 50 μg/mL of anti-IL6 antibody where indicated. Each experiment was performed in triplicate. Data are presented as the mean ± SEM. * indicates P<0.05 and ##, ** indicates P<0.01.

macrophages and treated with varying concentrations of PAI-1. We measured the levels of secreted immunosuppressive chemokines and cytokines that are downstream transcriptional target genes of NF-κB signaling, including CCL-17, CCL-22, and IL-6. We found that in both cell lines, low concentrations (5 and 15 nM) of PAI-1 treatment, in general, significantly increased the levels of secreted CCL-17, CCL-22, and IL-6 (Fig. 3A–F). In addition, higher concentrations (25, 35 and 45 nM) of PAI-1 increased CCL-17, CCL-22 and IL-6 expression

dose-dependently (Fig. 3A–F). We next measured the levels of secreted TGF-β after PAI-1 treatment, but found no significant change in secreted TGF-β levels, even with treatment with a high concentration of PAI-1 (Fig. 3G and H). However, when we measured levels of secreted TGF- β at 72 h post-PAI-1 treatment, the secretion of TGF-β was found to be increased significantly compared to the control group (Fig. 3I and J). We reasoned that the delay in the increase in secreted TGF- β levels was due to an IL-6-induced effect caused by TAM differentiation. When we blocked the effects of IL-6 by using an anti-IL-6 antibody, the secretion of TGF-β was effectively blocked (Fig. 3I and J).

TGF-β promotion effect of PAI-1 was via a TLR4 / NF-κB dependent pathway

To investigate the signaling pathways mediating the effect of PAI-1 on macrophages, we first investigated whether PAI-1 could activate the NF-κB by measuring IκBα phosphorylation at S65. We found that levels of phosphorylated IκBα increased even with low PAI-1 concentrations (5 and 15 nM) (Fig. 4A). We also knocked down TLR4 using siRNA, and found

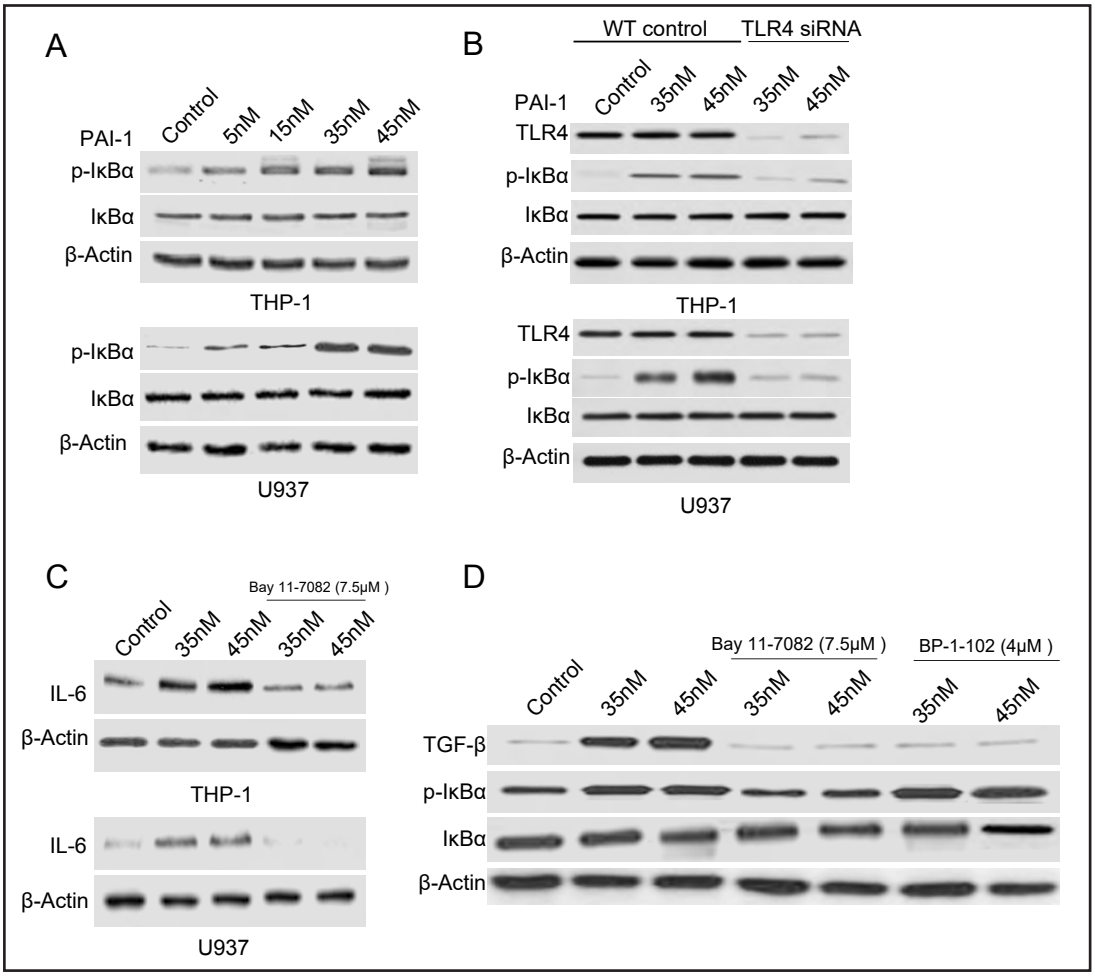

Fig. 4. TGF-β promotion effect of PAI-1 was via a TLR4 / NF-KB dependent pathway. A) Phosphorylated IκBα at S54 as well as total IκBα were assessed using western blot in THP-1- and U937-derived macrophages treated with varying PAI-1 concentrations. B) Wild-type and TLR4 knockdown THP-1- and U937-derived macrophages were treated with varying PAI-1 concentrations and phosphorylated IκBα at S54, total IκBα and TLR4 levels were assessed by western blot. C) IL-6 levels from THP-1- and U937-derived macrophages treated with PAI-1 and Bay 11-7082 were determined by western blot. D) TGF-β levels, phosphorylated IκBα at S54, as well as total IκBα were assessed using western blot in THP-1-derived macrophages treated with PAI-1, Bay 11-7082 and BP-1-102.

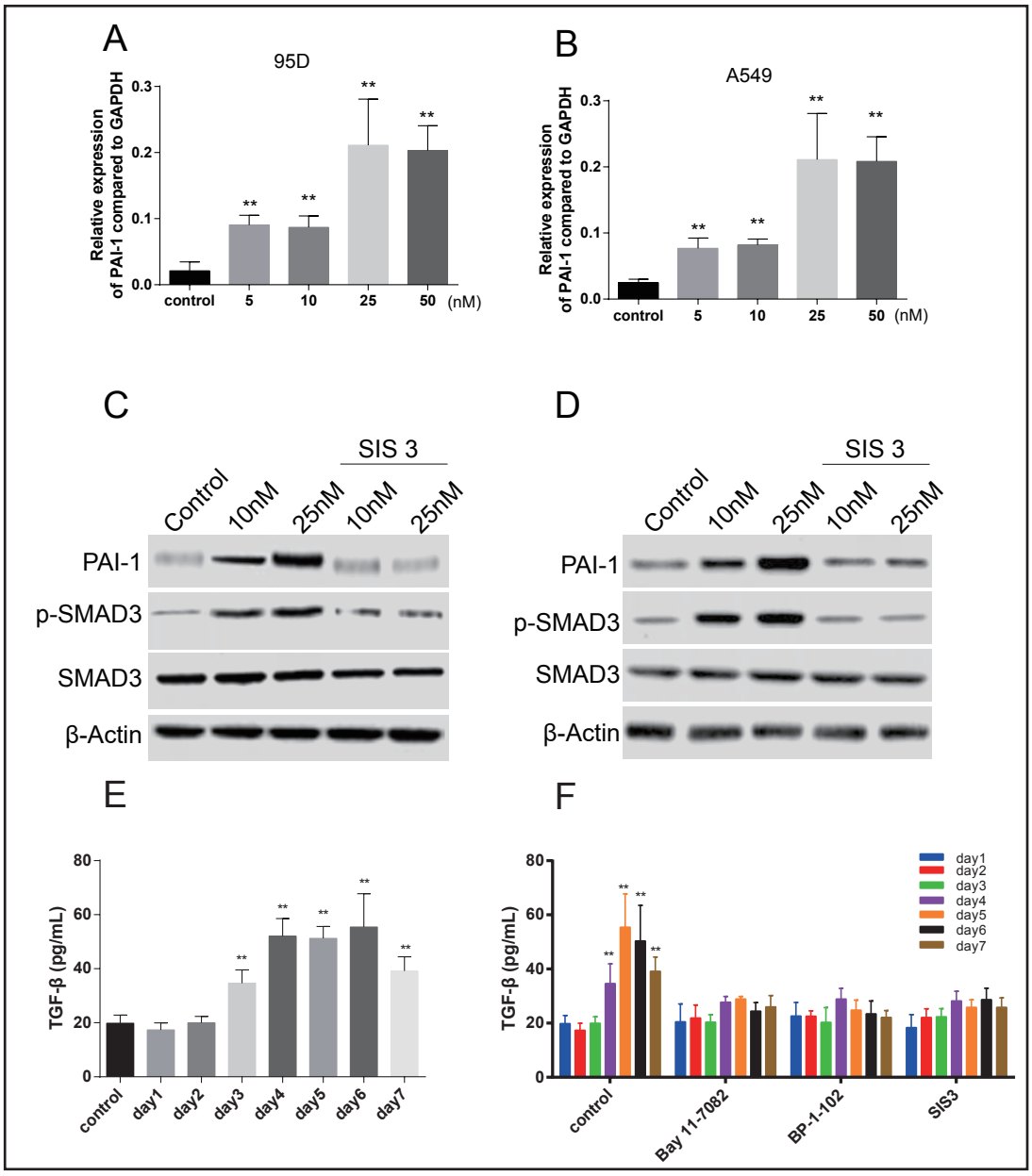

Fig. 5. TGF-β promotes PAI-1 expression in NSCLC cells and forms an immunosuppressive feed-forward loop. A, B) Levels of PAI-1 after treatment with varying concentrations of TGF-β in 95D and A549 NSCLC cells were determined using ELISA. C, D) Levels of PAI-1, phosphorylated SMAD3 at s425 as well as total SMAD3 were determined with western blot in 95D and A549 NSCLC cells after treatment with TGF-β and SIS3. E) Levels of secreted TGF-β were determined by ELISA in media from 3D culture systems treated with PAI-1. F) Levels of secreted TGF-β were determined by ELISA in the media of 3D culture systems treated with PAI-1 as control, Bay 11-7082, BP-1-102 and SIS3. Data are presented as the mean ± SEM. * indicates P<0.05 and ** indicates P<0.01.

that PAI-1 did not activate the NF-κB pathway effectively when TLR4 was downregulated in human macrophages (Fig. 4B). To investigate PAI-1-induced effects on the NF-κB pathway and IL-6 production, we used an NF-κB inhibitor (Bay 11-7082, 7.5 μM). IL-6 levels decreased significantly when NF-κB signaling was inhibited, even with treatment with high concentrations of PAI-1 (35 and 45 nM)(Fig. 4C). We then measured TGF-β production with treatment with Bay 11-7082 (7.5 μM) and with a STAT3 signaling inhibitor BP-1-102 (4 μM).

Both treatments inhibited the levels of secreted TGF-β in THP-1-derived macrophages, but TGF-β levels were markedly reduced with STAT3 inhibition even while NF-κB signaling was active (Fig. 4D). Based on these results, we postulated that PAI-1 promotes TGF-β signaling in macrophages by enhancing the NF-κB/IL-6/STAT3 pathway.

TGF-β can promote PAI-1 expression in NSCLC cells and formed an immunosuppression promoting fore forward loop

Based on previous studies, TGF-β promotes PAI-1 secretion in various cell types. Therefore, we investigated PAI-1 expression with treatment with varying concentrations of TGF-β in two NSCLC cell lines, 95D and A549. PAI-1 levels increased in both cell lines with TGF- β treatment in a dose-dependent manner (Fig. 5A, B). We then investigated changes in downstream signaling. SMAD3 phosphorylation and PAI-1 levels increased with increasing doses of TGF-β. However, secreted PAI-1 levels decreased with SMAD3 inhibition with SIS3 (Fig. 5C, D).

Since PAI-1 increases TGF-β secretion in macrophages, and TGF-β treatment can further increase PAI-1 production in NSCLC cells, it can be imagined that this feed-forward loop can contribute to cancer immunosuppression. To investigate this, we set up a 3D co-culture system with 95D and THP-1 cells. The microtissues were treated with PAI-1 (35 nM) and secreted TGF-β and PAI-1 levels were measured daily for 7 days. We found that secreted TGF-β levels did not significantly change within the first 2 days of treatment, but increased significantly after the third day. TGF-β levels reached a peak of about 55 ng/mL, with a subsequent decrease in levels thereafter (Fig. 5E). We treated this co-culture system with Bay 11-7082, BP-1-102 or SIS3 to inhibit NF-κ β, STAT3 and TGF-β signaling, respectively. All three inhibitors effectively blocked the increase in secreted PAI-1 and TGF-β levels in the microtissue cultures (Fig. 5F).

Discussion

TGF-β is a classical immunosuppressive cytokine that contributes to anti-inflammatory and immunosuppressive microenvironments. During acute inflammation, TGF-β is post-translationally regulated by being released from latency-associated protein, often by proteolysis. TGF-β further downregulates inflammation by targeting other immune cells such as regulatory T cells and type II macrophages [13]. Many previous reports have confirmed that excessive TGF-β existed with human NSCLC, and TGF-β has also regarded as a poor prognosis indicator for NSCLC [14-19]. However, these studies focused on increased TGF-β in regard to immune reactions and immune-regulation. Although the idea that the interaction between tumor and immune cells contributes to immunosuppression has been discussed for decades, recent studies addressing these issues are relatively rare. Our study demonstrates an interaction between tumor and immune cells, indicating that overexpression of tumor cell-derived PAI-1 is a causal factor in the overexpression of TGF-β in NSCLC.

PAI-1 is a risk factor of thrombosis and atherosclerosis due to its roles as a competitor of uPA [10]. Besides its roles in thrombosis and atherosclerosis, other roles related studies were relatively rare. However, studies indicated that PAI-1 is present in increased levels in various disease states (such as some forms of cancer), as well as in obesity and the metabolic syndrome [20-22]. In inflammatory conditions, PAI-1 appears to play a significant role in the progression to fibrosis, which is pathological formation of connective tissues. Presumably, lower PAI-1 levels would lead to increased fibrinolysis, or more rapid fibrin degradation. Recently, two studies have implicated an important role of PAI-1 in macrophages, with one study demonstrating that PAI-1 can regulate macrophage-dependent postoperative adhesion. The second study indicated that PAI-1 can activate macrophages through TLR4 [11, 23], which corroborates our observations that PAI-1 can activate NF-κβ signaling through TLR4. We saw an increase not only in pro-inflammatory cytokines such as IL-6, but also in anti-inflammatory factors such as TGF-β, CCL-17 and CCL-22, which are reported to

be downstream targets of NF-κβ signaling. CCL-17 and CCL-22 are two chemokines known to cause increased migration of negative regulators of inflammation to the tumor site [23]. IL-6 plays a pivotal role in the regulation of the tumor microenvironment. IL-6 increases the percentage of TAMs, which helps induce and exacerbate immunosuppression as well as promote angiogenesis through TGF-β and VEGF. In our study, secreted TGF-β increased PAI-1 expression in a TGF β R1/SMAD3-dependent fashion, forming a TGF- β -PAI-1 feed-forward loop between macrophages and NSCLC cells, respectively.

However, this loop has an auto-regulatory or auto-inhibitory mechanism that is affected by TGF- β concentration. Since TGF-β can inhibit NF-κB activation [24-26], when the levels of TGF-β induced by PAI-1 reach a certain concentration, the feed-forward loop would be inhibited. Subsequently, once the TGF-β concentration drops to a certain level, the NF-κB pathway would be reactivated and IL-6-induced TGF-β expression would increase again. We postulate this feed-forward loop is a sophisticated mechanism used in NSCLC to maintain TGF-β levels at an optimal concentration to avoid TGF- β -induced apoptosis-promoting effects, therefore maintaining immunosuppression in the microenvironment.

Conclusion

In summary, the present study provides evidence for a feed-forward loop induced by TGF-β and PAI-1, and controlled by NF-κB and STAT3 signaling in NSCLC, contributing to tumor immunosuppression. We think this loop may be a valuable therapeutic target for NSCLC treatment.

Disclosure Statement

No conflict of interests exists.

Acknowledgements

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