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Kinome-Wide Screening Identifies FAK as a Novel Post-Translational Regulator of PD-L1 Stability and Immune Evasion in Triple Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by limited treatment options and poor prognosis. Although immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have shown clinical promise, many TNBC patients exhibit resistance or limited response, underscoring the need to understand regulatory mechanisms of PD-L1 expression. Here, we performed a kinome-wide inhibitor screen using a HEK293A cell line stably expressing a NanoLuc-tagged PD-L1 construct lacking its endogenous promoter to identify post-translational regulators of PD-L1 stability. We identified focal adhesion kinase (FAK) as a novel modulator of PD-L1. FAK inhibition significantly decreased PD-L1 levels in HEK293A cells but paradoxically increased PD-L1 expression in TNBC cell lines. Mechanistically, FAK directly interacts with PD-L1 to modulate its stability independently of its kinase activity. Functionally, FAK inhibition enhanced membrane PD-L1 expression and reduced T-cell-mediated cancer cell killing, suggesting increased immune evasion. These findings reveal a novel role for FAK in immune modulation and suggest that combining FAK inhibitors with PD-L1 blockade may offer a promising strategy for TNBC treatment.

Keywords: breast cancer; PD-L1 stability; focal adhesion kinase; immunotherapy; immune evasion; kinome screen; kinase inhibitors

1. Introduction

Cancer immunotherapy has revolutionized the treatment landscape for various malignancies, offering new hope for patients with previously intractable diseases. Among the most promising approaches in this field is the targeting of immune checkpoint molecules, particularly the programmed death-ligand 1 (PD-L1) and its receptor, programmed cell death protein 1 (PD-1). The PD-1/PD-L1 axis plays a crucial role in maintaining immune homeostasis and preventing autoimmunity under normal physiological conditions. PD-L1 is overexpressed in cancer cells, which enhances their tumorigenic potential intrinsically (immune-independent)[1-5] or through immune evasion by evading immune surveillance via PD-1-mediated T cell inactivation [6-9]. Therefore, blocking inhibitory signaling by PD-1/PD-L1 with antibodies can suppress tumor growth by directly inhibiting tumor cell proliferation or reactivating cytotoxic T-cell behavior[7, 10, 11]. Many PD-1/PD-L1 blocking antibodies have been approved by the FDA for the treatment of multiple cancer types such as melanoma and lung cancer[11-13]. Over 2,000 PD-1/PD-L1 blockade clinical trials in various cancers are ongoing globally, and many patients are displaying impressive responses[14]. Therefore, anti-PD-1/PD-L1 immunotherapy is currently one of the most promising cancer therapies. Although durable responses have been achieved in melanoma and non-small cell lung cancer, objective response rates in breast cancer—particularly in triple-negative breast cancer (TNBC)—remain

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modest [15-17]. This underscores the need for a deeper understanding of the molecular mechanisms governing PD-L1 expression and regulation in cancer cells.

PD-L1 expression is regulated by cytokine signaling (e.g., IFN-γ/JAK-STAT), oncogenic pathways (PI3K/AKT/mTOR, Hippo/YAP/TAZ, MAPK) and post-translational modifications that govern protein stability and trafficking. Kinases orchestrate many of these processes and are highly druggable, yet only a few kinases including GSK3β, AMPK, ROCK and CK2 have been shown to act directly on PD-L1[18-20]. Although targeting PD-L1 or kinase involved in cancer alone have some clinical benefit in certain type of cancer, intrinsic or acquired resistance often arises after treatment. Recent studies strongly suggest that combined treatment of cancer with kinase inhibitors and anti-PD-L1 immunotherapy may overcome resistance of cancers to single drug therapy[21-27]. Therefore, identification of novel kinases interacting with PD-L1 may provide novel therapeutic strategy. We therefore undertook an unbiased kinome-wide screen to map new kinase regulators of PD-L1.

Using a HEK293A line that constitutively expresses NanoLuc (NL)-tagged PD-L1 devoid of its native promoter, we interrogated >500 kinases with selective inhibitors. This approach bypasses transcriptional inputs and focuses on post-translational control. The screen highlighted focal adhesion kinase (FAK) as a strong hit. FAK integrates integrin and growth-factor signaling to control adhesion, migration and survival[28]. FAK is frequently overexpressed in cancers including TNBC and is currently being tested in clinical trials[22, 23]. Yet its role in tumor immune evasion remains poorly defined. Here we delineate a previously unrecognized FAK–PD-L1 axis with therapeutic implications for TNBC.

2. Results

2.1. Establishment of Cell Line Stably Expressing NanoLuc Tagged PD-L1

To establish a stable cell line expressing a reporter for PD-L1 levels, HEK293A cells were transfected with a PD-L1-NanoLuc fusion (PD-L1-NL; Figure 1A) construct driven by the CMV promoter, followed by hygromycin selection. Single HEK293A clones expressing PD-L1-NL were isolated, expanded and subjected to luciferase assay and western blot analysis. As shown in Figure 1B-1C, the luciferase activity in each clone positively correlated with PD-L1-NL expression levels. To further validate our PD-L1 NanoLuc reporter, HEK293A-PD-L1-NL cells were treated with increasing concentration of GSK3β inhibitor, a well-known degrader of PD-L1 stability[29]. As expected, PD-L1-NL degradation increased in a dose-dependent manner, as evidenced by decreased luciferase activity and reduced PD-L1-NL protein levels (Figure 1D–1E). These results confirm the successful establishment of a HEK293A cell line stably expressing a NanoLuc-based PD-L1 reporter.

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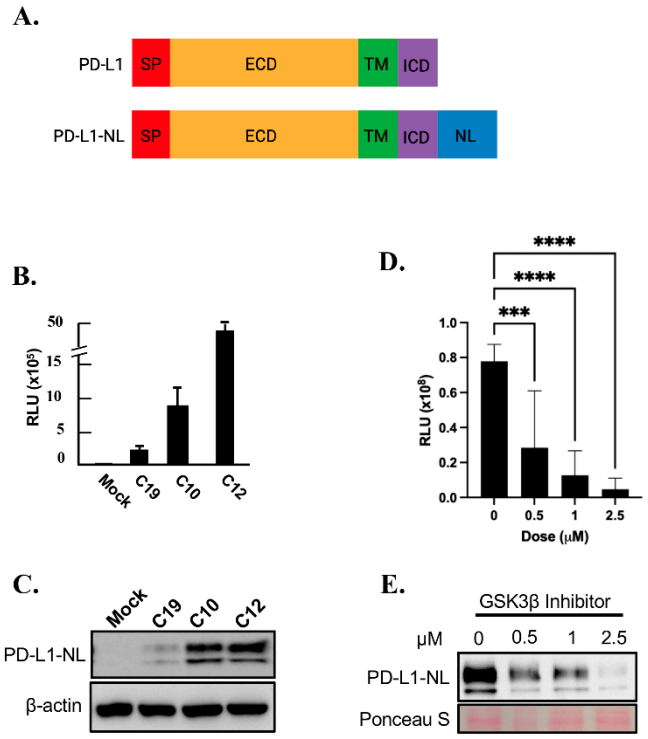

Figure 1. Establishing a HEK293A cell line stably expressing PD-L1-NL to monitor PD-L1 stability. **A**. Schematic representation of PD-L1 and PD-L1-NL construct. NL, NanoLuc; SP, signal peptide; ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. **B**. WB analysis of PD-L1-NL expression in different clones (C19, C10, C12) using anti-PD-L1 antibody. **C**. Luciferase activity of HEK293A clones (C19, C10, C12) expressing different levels of PD-L1-N. **D. E.** Dose-dependent reduction of PD-L1 stability by GSK3β inhibitor in HEK293A-PD-L1-NL cells. HEK293A-PD-L1-NL cells were treated at increasing concentration (0, 0.5, 1.0, 2.5 μM) of GSK3β inhibitor for 1 day, followed by luciferase assays (**D**) and western blot analysis (**E**). Statistical analysis for luciferase assays (**D**): ***, p<0.001; ****, p<0.0001. Ponceau S staining of proteins on membrane was used as protein loading control (**E**).

2.2. Kinome-Wide High Throughput Screen (HTS) for Kinase Inhibitors Regulating PD-L1 Stability

We next performed a kinome-wide HTS to identify kinase inhibitors that regulate PD-L1 stability. To minimize indirect effects associated with prolonged drug exposure, HEK293A-PD-L1-NL cells were treated with a library of 560 inhibitors targeting the human kinome for 24 hours, followed by luciferase assays (Figure 2A). Approximately 19 inhibitors were identified that reduced luciferase activity by more than twofold (Figure 2B; Table 1S). Among these, inhibitors targeting three previously known PD-L1 regulators including GSK3β, CK2, ROCK were confirmed[21, 22, 29], while 16 represented novel candidate regulators of PD-L1 stability. We have further validated the screening hits by luciferase assays. Six out of 10 kinase inhibitors (60%) tested are confirmed to suppress PD-L1-NL luciferase activity (Figure 2C). We further validated a subset of these hits via luciferase assays. Of the 10 inhibitors tested, 6 (60%) were confirmed to significantly suppress PD-L1-NL luciferase activity (Figure 2C). To determine whether the reduced luciferase signal reflected decreased PD-L1-NL protein levels, we treated HEK293A-PD-L1-NL cells with the validated inhibitors and performed western blot analysis. All tested inhibitors markedly reduced PD-L1-NL protein levels (Figure 2D). Taken together, these results identified six kinases, including three novel ones, CDK1/4/9, FAK, and ALK5, as regulators of PD-L1 stability.

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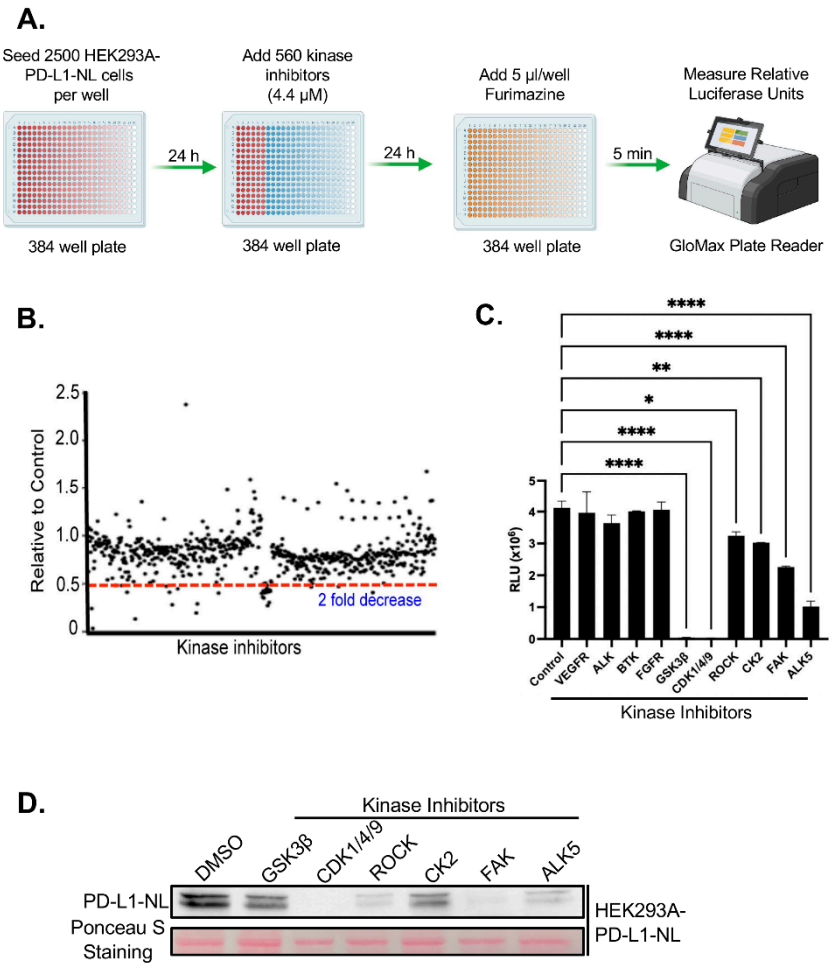

Figure 2. HTS for kinase inhibitors regulating PD-L1 stability. **A.** Flow diagram of kinome wide kinase inhibitor screening. **B.** Results of kinome-wide kinase inhibitors screening. The data represent ratios of relative light units (RLUs) of inhibitor-treated to those of DMSO control. **C.** Validation of potential kinase inhibitors using luciferase assay. Statistical analysis: *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$. **D**. Western blot analysis of PD-L1-NL expression in HEK293A cells. HEK293A-PD-L1-NL cells were treated with 5 μM for 1 day, followed by western blot analysis. Protein on the membrane was stained with Ponceau S as protein loading control.

2.3. Validation of FAK as Novel Regulator of PD-L1 Stability

Given the strong effect of FAK inhibitors on PD-L1 stability observed in our screen (Figure 2D) and the established role of FAK in TNBC [30], we further investigated whether FAK is a novel regulator of PD-L1. First, we validated that the FAK inhibitor PF431396 used in the HTS reduced PD-L1-NL luciferase activity and protein levels in HEK293A cells in a dose-dependent manner (Figure 3A). To rule out potential off-target effects of PF431396, we also treated HEK293A-PD-L1-NL cells with Defactinib, a more specific FAK inhibitor currently undergoing Phase II clinical trials[31]. Notably, similar to PF431396, Defactinib also decreased PD-L1-NL stability in a dose-dependent fashion (Figure 3B).

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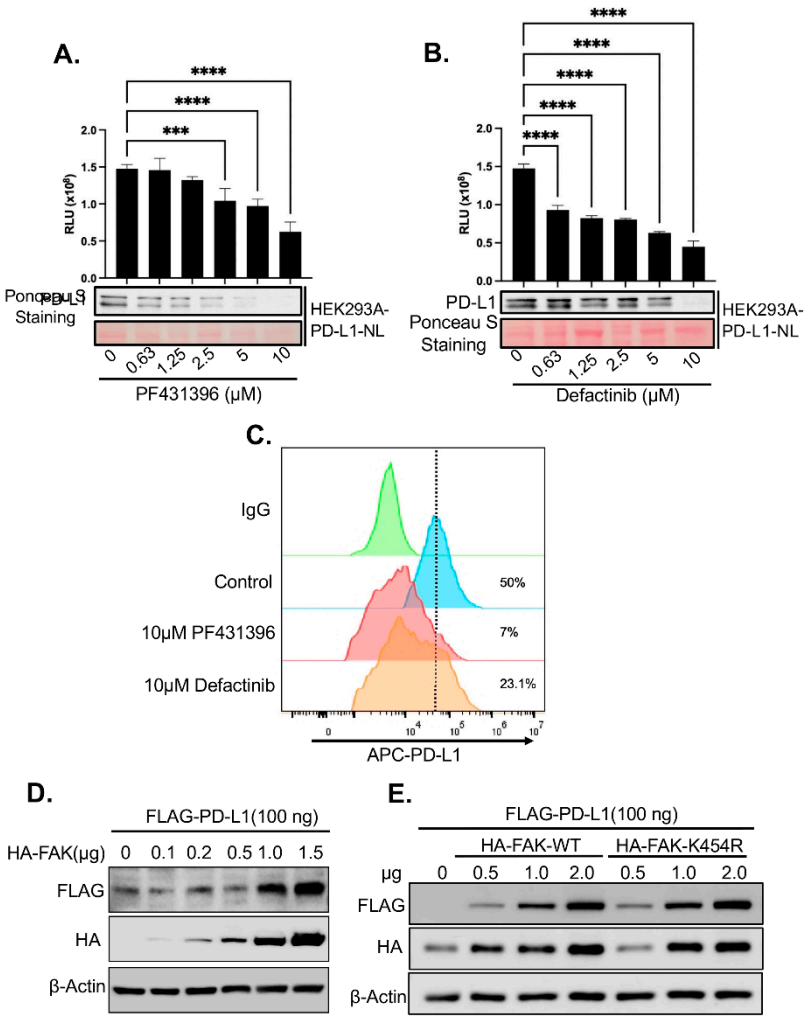

Figure 3. Validation of FAKi-induced PD-L1 degradation in HEK293A cells. **A**. Dose-dependent effect of FAKi PF431396 on PD-L1 stability. Lower panel: western blot analysis. Upper panel: luciferase assay. Statistical analysis: ***, p<0.001; ****, p<0.0001. **B**. Dose-dependent effect of FAKi Defactinib on PD-L1 stability. Lower panel: western blot analysis. Upper panel: luciferase assay. Statistical analysis: ****, p<0.0001. **C.** FACS analysis of the effect of various FAKi on membrane PD-L1. **D.** Dose-dependent effect of FAK on PD-L1 stability. **E.** Kinase activity independent effect of FAK on PD-L1 stability.

Since PD-L1 must localize to the cell membrane to engage PD-1 on immune cells and promote immune evasion, we next examined whether FAK inhibition affects membrane-bound PD-L1. Treatment with either PF431396 or Defactinib significantly reduced membrane PD-L1 levels in HEK293A cells (Figure 3C).

We then assessed the impact of FAK overexpression on PD-L1 levels. Co-transfection of increasing amounts of HA-tagged FAK together with a fixed amount of HA-tagged PD-L1 into HEK293A cells led to a dose-dependent increase in PD-L1 protein levels (Figure 3D). Surprisingly, this effect was independent of FAK's kinase activity, as a kinase-dead mutant (FAK-K454R) was equally effective at increasing PD-L1 stability compared to the wild-type (WT) protein (Figure 3E).

To determine whether FAK also regulates PD-L1 in TNBC cells, we treated two FAK- and PD-L1-positive TNBC cell lines, BT549 and Hs578T, with increasing concentrations of Defactinib. Unexpectedly, FAK inhibition in these cells led to elevated levels of both total and membrane-bound PD-L1 (Figures 4A–B and 5A–B), in contrast to the effects observed in HEK293A cells. To confirm that this upregulation was due to specific inhibition of FAK, we performed short interference RNA (siRNA)-mediated knockdown of FAK in both cell lines. Consistent with Defactinib treatment, FAK

 doi:10.20944/preprints202508.0162.v1

siRNA (siFAK) knockdown also significantly increased total and membrane PD-L1 levels in both TNBC cell lines (Figures 4C-4D, 5C-5D).

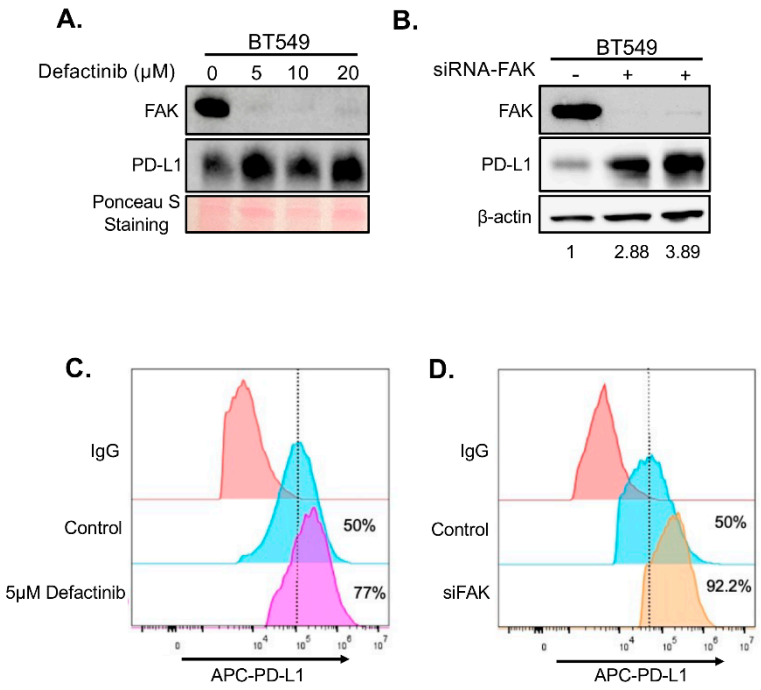

Figure 4. Loss of FAK increases PD-L1 stability in BT549 cells. **A**. Western blot analysis of PD-L1. BT549 cells were treated with 0, 5, 10, and 20 μM of Defactinib for 1 day. Protein on the membrane was stained with Ponceau S Stain. Stained protein was used as loading control. **B.** Western blot analysis of PD-L1 in BT549 cells after an siRNA-mediated FAK knockdown. **C.** FACS analysis of the effect of 5 μM of Defactinib on membrane PD-L1 in BT549 cells. β-actin was used as a control. Densitometry calculations were done using the adjusted band density calculated in ImageLab to compare the PD-L1 expression by dividing the adjusted band density of the siFAK samples by that of β-actin's adjusted density, resulting in the relative increase of PD-L1 protein expression. **D.** FACS analysis of the effect of siFAK on membrane PD-L1 in BT549 cells.

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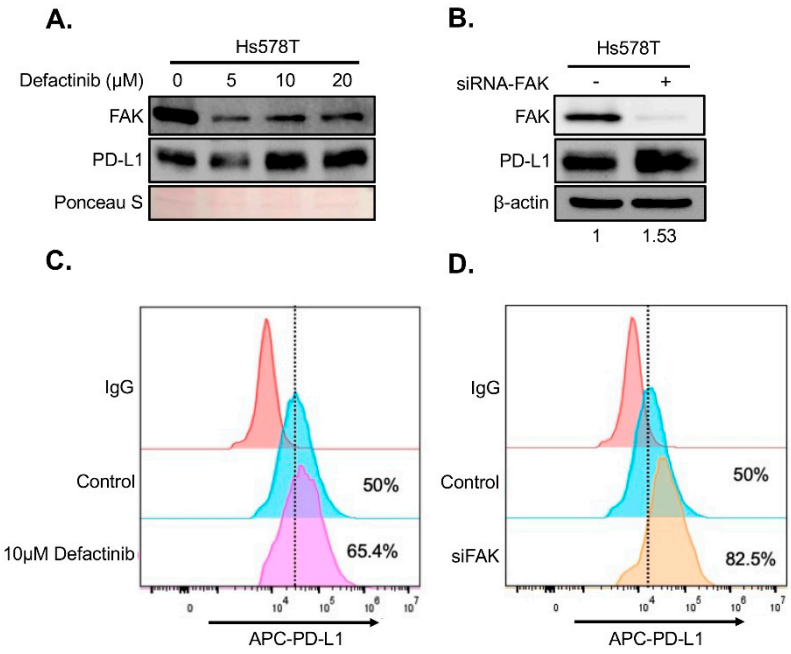

Figure 5. Loss of FAK increases PD-L1 stability in Hs578T cells. **A**. Western blot analysis of PD-L1 in Hs578T cells were treated with 0, 5, 10, and 20 μM of Defactinib for 1 day. Protein on the membrane was stained with Ponceau S. Stained protein was used as loading control. **B**. Western blot analysis of PD-L1 in Hs578T cells after an siRNA-mediated FAK knockdown. β-actin was used as a control. Densitometry analysis was as described in Fig. 4C legend. **C**. FACS analysis of the effect of 5 μM of Defactinib on membrane PD-L1 in Hs578T cells. **D**. FACS analysis of the effect of siFAK on membrane PD-L1 in Hs578T cells.

Since the PD-L1-NL construct lacks a promoter, changes in luciferase signal in HEK293A cells reflect post-transcriptional regulation. However, because TNBC cells retain the endogenous PD-L1 promoter, it remained unclear whether the increase in PD-L1 levels following FAK inhibition was due to enhanced transcription. To address this, we measured PD-L1 mRNA levels following FAK knockdown. Interestingly, siFAK also increases PD-L1 mRNA expression in both TNBC cell lines (Figure 6), suggesting that FAK can regulate PD-L1 at the transcriptional level in these cells as well.

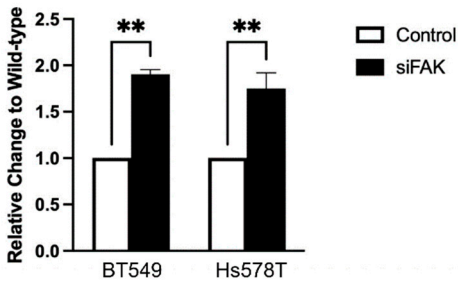

Figure 6. Quantitative Real-Time qRT-PCR analysis of TNBC cell lines after siRNA-mediated FAK knockdown. Relative change of PD-L1 RNA levels before (Wild-type) and after siFAK-mediated FAK knockdown (siFAK) in BT549 and Hs578T cells. Statistical analysis: **, $p<0.01$.

To further investigate whether FAK directly interacts with PD-L1 and regulates its stability, we examined the physical association between the two proteins *in vivo*. We transfected MYC-tagged FAK into HEK293A-PD-L1-NL cells, followed by protein extraction and co-immunoprecipitation (Co-IP) analysis. Immunoprecipitation using an anti-MYC antibody, but not IgG control, successfully pulled down PD-L1-NL (Figure 7A). Conversely, immunoprecipitation of PD-L1-NL using an anti-PD-L1

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antibody pulled down MYC-tagged FAK (Figure 7B). This interaction was further confirmed through co-transfection of FAK-MYC and PD-L1-FLAG into HEK293A cells (Figures 7C–7D).

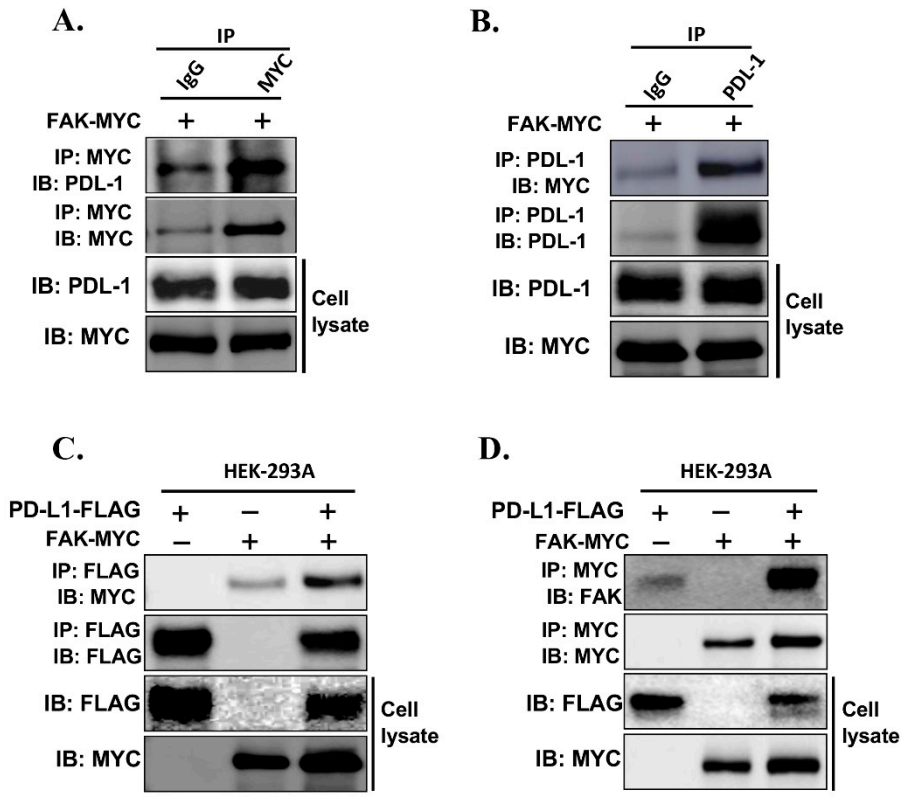

Figure 7. PD-L1 interacts with FAK *in vivo*. (**A and B**) PD-L1 interacts with FAK in vivo. PD-L1 stably overexpressed HEK-293A-pNLF1C-PDL-1 cells were transfected with MYC-tagged-FAK plasmids. The cells were harvested in 1% NP-40 lysis buffer. After checking the expression level of MYC-tagged-FAK or PD-L1, equal amount of cell lysates was subjected to co-immunoprecipitation assays using anti-rabbit-IgG or anti-MYC antibody (**A**) and anti-rabbit or anti-PD-L1 antibodies (**B**) respectively and immunoblotting analysis were performed using anti-PD-L1 or anti-FAK antibody respectively. (**C and D**) PD-L1 interacts with FAK *in vivo*. HEK-293A cells were transfected with MYC-tagged-FAK plasmids or SFB-tagged PD-L1 alone or together. The cells were harvested in 1% NP-40 lysis buffer. After checking the expression level of MYC-tagged-FAK or FALG-PD-L1, equal amount of cell lysates was subjected to co-immunoprecipitation assays using anti-FLAG antibody (**C**) or anti-MYC antibody (**D**) and immunoblotting analysis were performed using anti-MYC or anti-FLAG antibody, respectively.

Together, these results strongly support that FAK directly binds to PD-L1 and regulates its levels in cancer cells.

2.4. Upregulation of PD-L1 After FAK Inhibition Causes Increased Immune Evasion

Since FAK inhibition leads to elevated levels of membrane PD-L1, which may bind to PD-1 on T cells and promote immune evasion, we assessed whether FAK inhibition affects T cell–mediated killing of TNBC cells. Jurkat T cells were activated by treatment with IL2 and anti-CD3 antibody for 12 hours, resulting in high PD-1 expression (Figure 8A). As expected, co-culture of inactivated (PD-1-negative) Jurkat T cells with BT549 cells had no effect on cancer cell viability. In contrast, co-culture with activated (PD-1-positive) Jurkat T cells induced substantial cancer cell death (Figures 8B–8E). Importantly, inhibition of FAK, either by Defactinib treatment or siRNA-mediated knockdown (siFAK), significantly impaired T cell–mediated killing of BT549 cancer cells (Figures 8B–8E), indicating that FAK inhibition enhances immune evasion by upregulating PD-L1.

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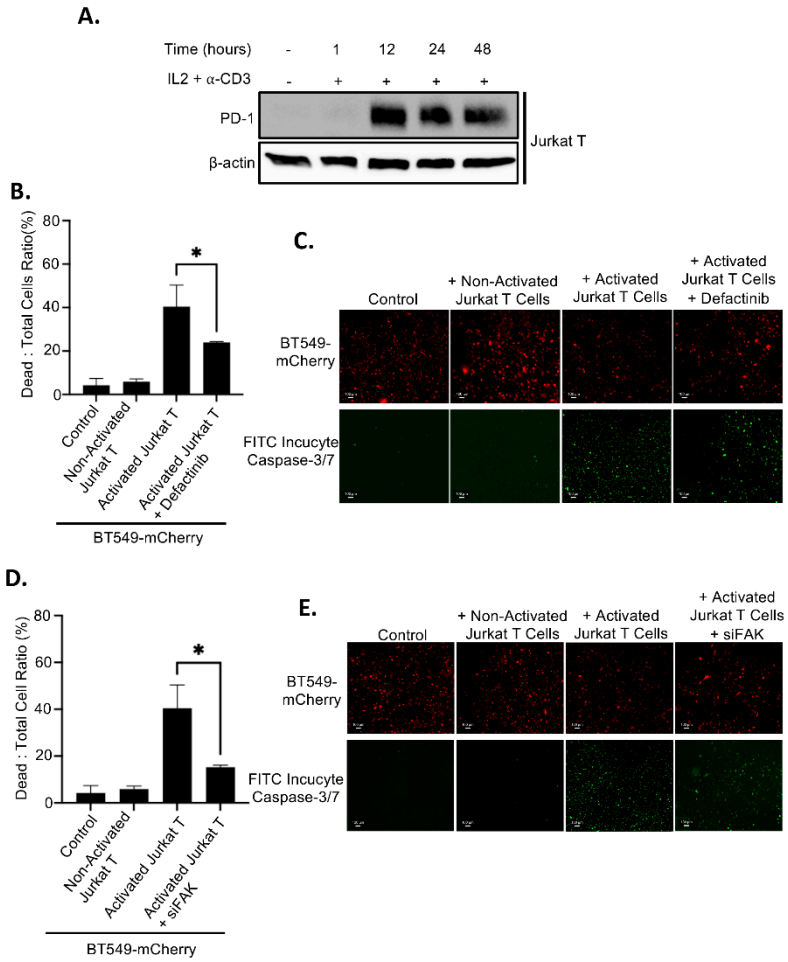

Figure 8. PD-L1 regulation by FAK regulates breast cancer immune evasion. **A.** Treatment of 20 ng/mL IL-2 and 200 ng/mL of anti-CD3 for 12, 24, and 48 hours induces the PD-1 expression in Jurkat T cells. **B.** Dead cells: total cells ratio of T cell killing at in wildtype and 5 μM Defactinib treatment in BT549-mCherry cells at a 2.5:1 effector to target cell ratio. Statistical analysis: *, p<0.01. **C.** Fluorescence of live cells (mCherry, red) and dead cells (FITC incucyte caspase– 3/7, green) in BT549-mCherry cells at a 4x magnification. **D.** Dead cells: total cells ratio of T cell killing at in wild-type siRNA-mediated FAK knockdown in BT549-mCherry cells at a 2.5:1 effector to target cell ratio. Statistical analysis: *, p<0.01. **E.** Fluorescence of live cells (mCherry, red) and dead cells (FITC incucyte caspase – 3/7, green) in BT549-mCherry cells at a 4x magnification.

3. Discussion

Although triple-negative breast cancer (TNBC) accounts for only 15–20% of all breast cancer cases, its high rates of metastasis and recurrence, poor prognosis, and limited treatment options pose significant clinical challenges, largely due to the lack of well-defined molecular targets. Therefore, the identification of novel diagnostic and therapeutic targets for TNBC is urgently needed.

FAK is a non-receptor protein tyrosine kinase known to promote tumorigenesis and metastasis by regulating key cancer hallmarks, including cell proliferation, survival, migration, and invasion **[31]**. Therefore, FAK has emerged as a potential therapeutic target in various cancers. Several small-molecule inhibitors targeting FAK's kinase function are currently under development and undergoing clinical testing**[32]**. Despite promising results in preclinical and early-phase trials, no FAK inhibitor has yet achieved clinical success. Notably, FAK is frequently upregulated in TNBC compared to normal breast tissue, supporting its candidacy as a therapeutic target in this aggressive subtype **[33]**. Elevated FAK expression in breast tumors is associated with aggressive features, including increased invasiveness and the triple-negative phenotype, reinforcing its prognostic value and therapeutic relevance**[34]**. Although FAK inhibitors have shown efficacy in suppressing TNBC

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cell invasion and tumor growth in preclinical xenograft models**[35, 36]**, their clinical application in TNBC has thus far been unsuccessful. The underlying reasons remain unclear. Our findings offer a potential explanation: FAK inhibition stabilizes PD-L1, which may enhance immune evasion in TNBC cells. This mechanism could contribute to the lack of clinical efficacy observed with FAK inhibitors as monotherapy. Therefore, combining FAK inhibitors with immune checkpoint blockade, such as anti-PD-L1 therapy, may represent a more effective strategy for TNBC treatment. Supporting this idea, a recent study reported that the PD-L1 inhibitor Atezolizumab acts synergistically with FAK inhibition to suppress TNBC cell invasion and motility**[35]**. Similarly, previous studies have shown that PARP inhibitors (PARPi) also upregulate PD-L1 expression and promote immunosuppression, and that combined PARPi and anti-PD-L1 therapy yields superior antitumor effects compared to either agent alone**[37].** Together with our findings, these results highlight a broader mechanism whereby targeted therapies may inadvertently promote immune escape through PD-L1 upregulation. Thus, combination strategies that integrate both targeted and immune therapies may provide a promising approach to overcome intrinsic or acquired resistance in TNBC and other cancers.

Our studies identified 3 novel kinases including FAK, CDK1/4/9, and ALK5 as novel kinase regulators of PD-L1 stability in HEK293A cells after HTS. Further validation also shows that CDK1/4/9 inhibitor instead of ALK5 inhibitor significantly reduce PD-L1 levels in TNBC cells (unpublished). It will be very interesting to further explore whether CDK1, CDK4, or CDK9 regulates PD-L1 stability in immune evasion of TNBC cells.

While our discovery of the FAK–PD-L1 signaling axis in immune evasion is novel and promising, several questions remain unanswered. First, the opposing effects of FAK inhibition, decreasing PD-L1 levels in HEK293A cells but increasing them in TNBC cells, are not yet understood. It is possible that differential expression of cofactors, phosphatases, or ubiquitin ligases between cell types influences FAK-mediated PD-L1 regulation. Therefore, it is important to check the effects of FAK inhibition on PD-L1 stability first when other types of cancer are treated with FAK inhibitors alone and in combination with anti-PD-L1 immunotherapy. Second, although we confirmed a direct interaction between FAK and PD-L1 in cancer cells via co-immunoprecipitation, the mechanistic basis of this interaction remains unclear. PD-L1 is a membrane protein, while FAK is predominantly cytoplasmic. However, FAK has been shown to interact with the intracellular domains of membrane proteins such as integrin and E-cadherin [26], suggesting that FAK may similarly bind the intracellular region of PD-L1. Notably, other kinases known to regulate PD-L1 stability, such as GSK3β, CK2, and ROCK, are also localized in the cytoplasm [22-24]. Therefore, it is plausible that FAK interacts with PD-L1 in the cytoplasm during its processing, thereby destabilizing it and preventing membrane localization. In contrast, inhibition of FAK may stabilize PD-L1 and promote its translocation to the membrane. in our studies. Finally, our current study was conducted entirely *in vitro* using cancer cell lines. It remains to be determined whether FAK inhibition promotes immune evasion and tumor progression *in vivo*. Future studies using immunocompetent syngeneic mouse models, such as 4T1/Balb/c, will be essential to evaluate whether combined treatment with FAK inhibitors and anti-PD-L1 antibodies offers superior efficacy against metastatic TNBC.

4. Materials and Methods

4.1. Kinase Inhibitors

CIHR99021 (GSK3 inhibitor), SU4312 (VEGFR inhibitor), Asp-3026 (ALK inhibitor), PCI-32765 (BTK inhibitor), BGJ398 (FGFR inhibitor), Y39983 (ROCK inhibitor), P276-00 (CDK1/4/9 inhibitor), PF431396 and Defactinib (FAK inhibitor), and CX-4945 (CK2 inhibitor) were purchased from TargetMol. SJN2511 (ALK5 inhibitor) was purchased from Cayman Chemical.

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4.2. Plasmid Construction

For cloning PD-L1 open reading frame (ORF) into pNLF1-C [CMV/hygro] vector expressing NanoLuc at C-termini, PD-L1 is amplified by PCR (EcoRI-PD-L1F: 5′-GCTGAATTCA CCATGAGGATATTTGCTGTCTT; EcoRV-PD-L1R-no-stop: 5′-ACAGATA TCG TCTC CTCCAAATGTGTATC-3′). The PCR product was cut by EcoRI/EcoRV and subsequently cloned into the EcoRI/EcoRV sites of pNLF1-C vector (Promega) expressing NanoLuc at C-terminal of protein.

4.3. Cell Culture and Establishment of Stable Line

HEK293A (human embryonic kidney cells), HEK293T, and Hs578T were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma D6429). BT549 was cultured in Roswell Memorial Institute-1640 (RPMI-1640; Sigma R8758). All above mentioned cells were cultured in medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (Invitrogen) as antibiotics.

For establishment of cell line stably expressing PD-L1-NL, HEK293A cells were transiently transfected with PD-L1/pNLF1-C, followed by hygromycin (400 μg/ml) treatment and clonal selection. Different clones were isolated, expanded, and subjected to western blot analysis and luciferase assays.

4.4. Kinome-Wide Kinase Inhibitor Screen and Drug Validation

Kinome-wide kinase inhibitor screen is as described previously [20]. HEK293A-PD-L1-NL cells were used in the screen.

4.5. NanoLuc Luciferase Assay

Approximately 1 × 10^4 HEK293A-pNLF-PD-L1-C20 cells were seeded in triplicate into each well of a 96-well plate 24 hours prior to treatment. Cells were then treated with increasing concentrations of the indicated drugs. After 24 hours, Nano-Glo® Live Cell Reagent (Promega), containing the cell-permeable furimazine substrate, was added directly to each well. Luminescence was immediately measured using a Promega™ GloMax® Plate Reader.

4.6. RNA Extraction and qRT-PCR

RNA extraction, quantification, and qRT-PCR are as described[20]. For internal control, 18S rRNA was used. The following primers are used in qPCR: rRNA, sense: 5′-TCCCCATGAACGAGGAATTCC-3′, anti-sense:5′-AACCATCCAATCGGTAGTAGC-3′; PD-L1, sense: 5′-GGCATTTGCTGAACGCAT-3′, anti-sense:5′-CAATTAGTGCA GCCAGGT-3′.

4.7. Knockdown of FAK by Short Interference RNA (siRNA)

Knockdown of FAK by siRNA were performed as described as previously[38] siFAKs were purchased from IDT. siFAK sequences are the following: siFAK-1: sense, 5′-AAGCCAACATTGAAUUUCUUCUATC-3′; anti-sense, 5′-GAUAGAAGAAAUUCA AUGUUGGCUUAU-3′; siFAK-2: 5′-AAGACAGTACTTACTATAAAGCTTC-3′; anti-sense: 5′-GAAGCTTTAUAGTAAGTACTGTCTCC-3′.

4.8. Immunoblotting, co-Immunoprecipitation (Co-IP), and Antibodies

Immunoblotting and Co-IP were carried out as described previously[21]. Briefly, cells grown to 70% to 80% confluency were harvested in RIPA/1% NP-40 lysis buffer. Protein samples were subjected to SDS-PAGE and immunoblotted with respective antibodies using standard protocols. For Co-IP, cells expressing different genes were harvested in 1% NP-40 lysis buffer. After checking the expression of different proteins, equal amounts of proteins were precleared overnight at 4°C using Protein A/G-agarose. The supernatant was subjected to immunoprecipitation using anti-HA-F7 or

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FLAG-M2 antibodies for 2 hours at 4°C. After the incubation, 20 µl of Protein A/G-agarose was added for an additional 1 hour. The beads were washed four times with 150 mM NaCl-1% NP-40 lysis buffer, suspended in 20 µl of 2x SDS sample buffer, boiled for 5 minutes, and centrifuged. The supernatants were run on SDS-PAGE and blotted with respective antibodies. The antibodies used for Western blot analysis were as follows: PD-L1 (E1L3N), anti-FLAG-M2, and β-actin from MilliporeSigma (Etobicoke, ON, Canada); antibodies against HA-F7 and FAK (D-1) from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal anti-Myc (9E10) from Roche (Mississauga, ON, Canada). Densitometry calculations were done using the adjusted volume calculated in the ImageLab 6.1 software (Bio-Rad) and dividing the sample adjusted volume to β-actin's adjusted volume resulting in the relative increase of PD-L1 protein expression.

4.9. Flow Activating Cell Sorting (FACS) Analysis of Membrane PD-L1

FACS analysis of membrane PD-L1 was conducted following a standard protocol provided by Abcam. A PE-conjugated anti-human PD-L1 antibody (clone MIH1, eBioscience) was used for detection, with mouse IgG1 (clone B11/6, Abcam) serving as the isotype control. Briefly, 1×10^6 cells were resuspended in 100 µL of staining buffer containing either the control IgG or APC-conjugated anti-PD-L1 antibody (diluted 1:100), and incubated at room temperature for 30 minutes on a rotator in the dark. After staining, cells were washed three times with 250 µL of staining buffer. Following centrifugation, the cell pellets were resuspended in 0.5 mL of Cell Staining Buffer, filtered through a 35 µm cell strainer (VWR), and maintained on ice in the dark until analysis. Flow cytometry was performed using a Beckman Coulter CytoFlex S instrument.

4.10. Immune Evasion Assays In Vitro

To enable co-culture assays, BT549 cells were labeled with red fluorescence via infection with a lentivirus expressing mCherry. Jurkat T cells were activated by treatment with anti-CD3 antibody (200 ng/mL) and IL-2 (20 ng/mL) for 12 hours to induce PD-1 expression prior to co-culture. Triplicates of 2×10^4 cancer cells were seeded into each well of a 96-well plate. After overnight incubation, approximately 2×10^6 activated Jurkat T cells were added to each well containing cancer cells, followed by the addition of Incucyte® Caspase-3/7 Apoptosis Reagent (Green dye, 5 µM; Sartorius, Cat#4440). Cancer cell killing was monitored and quantified by immunofluorescence imaging over 24 hours using the CellCyte system (CYTENA). Red fluorescence indicated live cancer cells, while green fluorescence indicated apoptotic (dead) cells. The percentage of green (dead) cells relative to the total cell population (green + red) was calculated along with standard deviation.

Statistical Analysis

All statistical analysis were performed using the Prism 10 program.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: title; Table S1

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.Y.; methodology, A.B., P.K., T.K., Y.H.; formal analysis, A.B., P.K., T.K., Y.H.; investigation, A.B., P.K, T.K., Y.H.; writing—original draft preparation, A.B., X.Y.; writing—review and editing, A.B; supervision, X.Y.; project administration, Y.H.; funding acquisition, X.Y. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
APC	Allophycocyanin (fluorescent dye)
BTK	Bruton's Tyrosine Kinase
CDK	Cyclin-Dependent Kinase
CK2	Casein Kinase 2
Co-IP	Co-immunoprecipitation
DMEM	Dulbecco’s Modified Eagle Medium
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal Adhesion Kinase
FDA	U.S. Food and Drug Administration
FGFR	Fibroblast Growth Factor Receptor
GSK3	Glycogen Synthase Kinase 3
HA	Hemagglutinin (epitope tag)
HEK293A	Human Embryonic Kidney 293A cells
HTS	High Throughput Screening
ICI	Immune Checkpoint Inhibitor
IL2	Interleukin 2
MAPK	Mitogen-Activated Protein Kinase
NL	NanoLuc
NP-40	Nonidet P-40 (detergent)
NanoLuc	NanoLuciferase
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PDX	Patient-Derived Xenograft
PE	Phycoerythrin (fluorescent dye)
PI3K	Phosphoinositide 3-Kinase
RIPA	Radioimmunoprecipitation Assay buffer
ROCK	Rho-associated Protein Kinase
TAZ	Transcriptional coactivator with PDZ-binding motif
TNBC	Triple Negative Breast Cancer
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild-Type
mTOR	Mechanistic Target of Rapamycin
qRT-PCR	Quantitative Real-Time PCR
siFAK	siRNA targeting FAK
siRNA	Small Interfering RNA

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