NEK2 inhibition triggers anti-pancreatic cancer immunity by targeting PD-L1

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Despite the substantial impact of post-translational modifications on programmed cell death 1 ligand 1 (PD-L1), its importance in therapeutic resistance in pancreatic cancer remains poorly defined. Here, we demonstrate that never in mitosis gene A-related kinase 2 (NEK2) phosphorylates PD-L1 to maintain its stability, causing PD-L1-targeted pancreatic cancer immunotherapy to have poor efficacy. We identify NEK2 as a prognostic factor in immunologically “hot” pancreatic cancer, involved in the onset and development of pancreatic tumors in an immune-dependent manner. NEK2 deficiency results in the suppression of PD-L1 expression and enhancement of lymphocyte infiltration. A NEK binding motif (F/LXXS/T) is identified in the glycosylation-rich region of PD-L1. NEK2 interacts with PD-L1, phosphorylating the T194/T210 residues and preventing ubiquitin-proteasome pathway-mediated degradation of PD-L1 in ER lumen. NEK2 inhibition thereby sensitizes PD-L1 blockade, synergically enhancing the anti-pancreatic cancer immune response. Together, the present study proposes a promising strategy for improving the effectiveness of pancreatic cancer immunotherapy.
Pancreatic ductal adenocarcinoma (PDAC) is the most common exocrine cell tumor of the pancreas and the 7th leading cause of death in cancer patients worldwide\(^1,2\). Despite advances in diagnosis, perioperative management, radiotherapy techniques, and systematic treatments that enhance the survival rate of pancreatic cancer, the numbers of such cases and related deaths have steadily increased\(^1,3\). Immunotherapy is considered the most promising strategy to reverse this trend, especially immune checkpoint blockade targeting programmed cell death 1 (PD-1) and its ligand 1 (PD-L1, also known as B7-H1)\(^4,5\). Multiple clinical trials of PD-1/PD-L1 blockade have been launched as treatments for melanoma, lung cancer, and kidney cancer, with promising clinical results\(^6-7\). However, PD-1/PD-L1 blockade has so far demonstrated limited effectiveness in PDAC. For example, Royal et al. reported that no in-group patients with advanced pancreatic cancer responded, preventing an evaluation of the effectiveness of anti-PD-L1 antibodies\(^8\). Furthermore, a phase II randomized clinical trial of 65 patients with metastatic PDAC suggested that the objective response rate of patients receiving durvalumab monotherapy was 0%, although that of patients receiving combination therapy was 3.1%\(^9\). Thus, an in-depth understanding of the detailed mechanism of PD-L1-based combinatorial therapy may contribute to the development of novel therapeutic strategies\(^10\).

Accumulating evidence has demonstrated that the therapeutic efficacy of drugs targeting PD-L1 can be seriously affected by post-translational modifications (PTMs), including ubiquitination, phosphorylation, and glycosylation. Such PTMs play critical roles in regulating the stability, internalization, and localization of PD-L1, in addition to its physiological and pathological functions\(^11\). For instance, N-glycosylation of PD-L1 facilitates its folding, intracellular transport, and function\(^12,14\). Epidermal growth factor (EGF) signaling upregulates b-1,3-N-acetylglucosaminyltransferase (B3GNT3) expression that promotes N-glycosylation at positions N192, N200, and N219 of PD-L1 in triple-negative breast cancer cells. This stabilizes PD-L1 through the prevention of glycan synthase kinase 3 beta (GSK3β) binding and phosphorylation of PD-L1 at the T180 and S184 residues, subsequently recognized by beta-transducin repeat containing E3 ubiquitin-protein ligase (BTRCP)-mediated PD-L1 poly-ubiquitination and degradation\(^15,16\). More importantly, the combination of EGFR inhibition and PD-L1 blockade significantly enhances mono-drug therapeutic efficacy. In addition, metformin-activated AMP-activated protein kinase directly phosphorylates PD-L1 at the S195 residue, inducing abnormal PD-L1 glycosylation that leads to PD-L1 degradation through an endoplasmic reticulum-associated protein degradation pathway\(^16\). Therefore, identification of critical regulators of PD-L1 PTM in pancreatic cancer is urgently required to improve the efficacy of PD-L1 blockade in this specific cancer type.

Never in mitosis gene A (NIMA)-related kinase 2 (NEK2) is a member of NEKs, a serine-threonine kinase family including proteins structurally related to the essential mitotic regulator\(^12\). NEK2 is a multifunctional protein physiologically involved in cell cycle regulation, such as centrosome duplication and separation\(^7,18\), microtubule stabilization\(^19\), kinetochore attachment\(^20\), in addition to spindle assembly checkpoint\(^21,22\). NEK2 is also considered a key factor in the maintenance of normal development and function of B cells\(^23\). Intriguingly, aberrant expression of NEK2 and NEK2-mediated phosphorylation of downstream proteins (e.g. p53 at Ser315 and GAS2L1 at Ser352) are frequently observed to be associated with tumor initiation, progression, metastasis, and consequently poor prognosis in multiple cancers, including but not limited to, breast cancer\(^26,27\), colorectal cancer\(^28\), and PDAC\(^29\). However, the potential role of NEK2 in cancer immune resistance so far remains undefined.

In this work, we demonstrate that PD-L1 is a substrate of NEK2, and combinatorial inhibition of NEK2 and PD-L1 largely improves the therapeutic efficacy of pancreatic cancer in pre-clinical models.

**Results**

**NEK2 is a prognostic factor in pancreatic cancer.** To assess whether members of the NEK family (from NEK1 to NEK11) are associated with pancreatic cancer prognosis, we first compared their expression levels in tumors and normal tissue using pancreatic adenocarcinoma (PAAD) datasets from The Cancer Genome Atlas (TCGA) database. Bioinformatic analyses across multiple cancers demonstrated that NEK2 is the most significantly upregulated member of the NEK family in PAAD compared with expression in normal tissues (Fig. 1a and Supplementary Fig. 1a). The elevated expression of NEK2 was further validated in paired PDAC tissue samples by immunohistochemistry (Fig. 1b, c). More importantly, the significantly negative prognostic relevance of NEK2 was observed in a tumor tissue microarray (Fig. 1d). The analysis of TCGA data further confirmed that the higher expression of NEK2 was correlated with inferior overall survival (OS) (Fig. 1e and Supplementary Fig. 1b). Furthermore, high expression of NEK2 was also found to be significantly associated with vascular invasion and tumor grade as opposed to TMB stage and other clinical indices in multiple patient cohorts of pancreatic cancer (Table 1 and Supplementary Fig. 2a–c). Together, these results suggest that the elevated expression of NEK2 is unfavorable for patients with PDAC.

**NEK2 expression indicates poor prognosis in immunologically “hot” pancreatic cancer.** Since tumors are classified as immunologically “cold” (CD8⁺ T cell-decreased) or “hot” (CD8⁺ T cell-enriched), and their immune status is closely associated with tumor prognosis\(^30\), we further investigated the prognostic role of NEK2 in increased or decreased CD8⁺ T cell pancreatic cancer. Intriguingly, NEK2 expression was only significantly associated with OS in immunologically “hot” cells, rather than those that were “cold” (Fig. 1f, g). Furthermore, the opposite relationship was observed between the expression of NEK2 and the number of immune effector cells (Fig. 1h). Interestingly, NEK2 was also expressed in multiple immunosuppressive populations, such as myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and macrophages, although the expression-positive rate was lower than that in tumor cells (Supplementary Fig. 3a, b). Furthermore, GO-enrichment analysis revealed that NEK2 overexpression led to a dramatic increase in the expression of proteins related to the positive regulation of leukocyte proliferation, migration, and activation, and lymphocyte activation (Supplementary Fig. 3c, d). These results indicate that NEK2 may influence the efficacy of immunotherapy in multiple ways. Additionally, given the critical role of tumor mutation burden (TMB) in the immunotherapy response, we next investigated the prognostic relevance of NEK2 in immunologically “hot” pancreatic cancer with high or low TMB, respectively. Surprisingly, NEK2 displayed a significant correlation with poor prognosis in low mutational tumors as opposed to high mutational tumors, further indicating the precise prognostic value of NEK2 in specific patients (Supplementary Fig. 4a, b). Taken together, NEK2 was found to be a prognostic factor in immunologically “hot” pancreatic cancer.

**NEK2 deficiency improves pancreatic cancer immunogenicity.** A KPC-NEK2 knockdown (KD) pancreatic cancer cell line, stably transfected with NEK2 double nickase plasmids, was generated to determine whether NEK2 is associated with an anti-tumor immune response. In comparison with wild-type (WT) control
cells, NEK2 KD-cells displayed significantly weakened resistance to T cell-mediated tumor cell-destruction in vitro (Supplementary Fig. 5a, b). The growth of NEK2 KD cell-derived tumors was consistently significantly repressed in immunocompetent C57BL/6 mice but not in immunodeficient nude mice (Fig. 2a–h). Flow cytometry further revealed a significant increase in the number and function of CD8+ T cells in NEK2 KD cell-derived tumors (Fig. 2i, j). Furthermore, immunocompetent mice with such tumors exhibited markedly extended survival that was not observed in immunodeficient nude mice (Fig. 2k–m). Taken together, the results suggest that NEK2 is involved in the development and progression of PDAC in an immune-dependent manner, with deletion of NEK2 improving pancreatic cancer immunogenicity.
Fig. 1 NEK2 is a prognostic factor for immunologically “hot” pancreatic cancer. a–c Expression profile of NEK2 in pancreatic cancer. Relative NEK2 expression in pancreatic cancer was analyzed using large-scale RNA-Seq datasets of PDAC from the TCGA database (n (T) = 179, n (N) = 177; n: number of patients) (a). NEK2 expression was measured in paired tumor and normal pancreatic tissues by IHC staining, with representative images (b) and statistical results (c) shown (n = 30; n: number of patients) (N: Normal pancreatic tissue; T: Pancreatic tumor tissue). Scale bars: 100 μm. d Correlation of NEK2 with prognostic factors in pancreatic cancer. Tissue microarray analysis of the prognostic role of NEK2 in pancreatic cancer (n = 64 weakly positive; n = 50 strongly positive) (p < 0.0001). e Overall survival (OS) of patients with pancreatic cancer with high or low expression of NEK2 (n = 177). f Overall survival (OS) of pancreatic cancer patients with high numbers of CD8+ T cells and with high or low expression of NEK2 (n = 76). g Overall survival (OS) of pancreatic cancer patients with decreased CD8+ T cells and with high or low expression of NEK2 (n = 101). h Bioinformatics analysis of the correlation between NEK2 and immune effector cells using TCGA datasets. In a, data are represented as boxplots where the middle line is the median; the lower and upper hinges correspond to the first and third quartiles; the upper whisker extends from the hinge to the largest value no further than 1.5 × IQR from the hinge (where IQR is the inter-quartile range); the lower whisker extends from the hinge to the smallest value at most 1.5 × IQR of the hinge, while data beyond the end of the whiskers are outlying points that are plotted individually. *P < 0.05, **P < 0.01, ***P < 0.001 using a two-tailed t-test; ns: not significant. Kaplan-Meier method and a Gehan-Breslow-Wilcoxon test are indicated in d. The Hazard Ratios (HR) and p-values by the log-rank (Mantel-Cox) test are indicated in e–g.

| Variable                      | NEK2 Low (H-score ≤ 130) | NEK2 High (H-score > 130) | P value |
|-------------------------------|--------------------------|---------------------------|---------|
| Gender                        |                          |                           | 0.925   |
| Male, n (%)                   | 44 (57.9)                | 44 (57.1)                 |         |
| Female, n (%)                 | 32 (42.1)                | 33 (42.9)                 |         |
| Age, years                    |                          |                           | 0.461   |
| >60, n (%)                    | 64 (84.2)                | 68 (88.3)                 |         |
| ≤60, n (%)                    | 12 (15.8)                | 9 (11.7)                  |         |
| BMI, kg/m²                    |                          |                           | 0.238   |
| <18.5, n (%)                  | 16 (22.2)                | 9 (12.7)                  |         |
| 18.5–23.9, n (%)              | 35 (48.6)                | 43 (60.6)                 |         |
| >23.9, n (%)                  | 21 (29.2)                | 19 (26.8)                 |         |
| TNM stage                     |                          |                           | 0.239   |
| I, n (%)                      | 14 (18.4)                | 11 (14.5)                 |         |
| II, n (%)                     | 40 (52.6)                | 48 (63.2)                 |         |
| III, n (%)                    | 19 (25.0)                | 11 (14.5)                 |         |
| IV, n (%)                     | 3 (4.0)                  | 6 (7.9)                   |         |
| Vascular invasion             |                          |                           | 0.043   |
| Yes, n (%)                    | 30 (39.0)                | 42 (55.3)                 |         |
| No, n (%)                     | 47 (61.0)                | 34 (44.7)                 |         |
| Nerve invasion                |                          |                           | 0.086   |
| Yes, n (%)                    | 48 (63.2)                | 57 (76.0)                 |         |
| No, n (%)                     | 28 (36.8)                | 18 (24.0)                 |         |
| Serum CA12-5, U/ml            |                          |                           | 0.427   |
| ≥35, n (%)                    | 19 (29.2)                | 17 (23.3)                 |         |
| <35, n (%)                    | 46 (70.8)                | 56 (76.7)                 |         |
| Serum CA19-9, U/ml            |                          |                           | 0.907   |
| ≥37, n (%)                    | 63 (81.8)                | 60 (81.1)                 |         |
| <37, n (%)                    | 14 (18.2)                | 14 (18.9)                 |         |
| Serum CEA, U/ml               |                          |                           | 0.714   |
| ≥5, n (%)                     | 24 (35.8)                | 24 (32.9)                 |         |
| ≤5, n (%)                     | 43 (64.2)                | 49 (67.1)                 |         |
| Tumor differentiation         |                          |                           | 0.045   |
| Well, n (%)                   | 3 (5.6)                  | 5 (6.8)                   |         |
| Moderate, n (%)               | 53 (72.6)                | 39 (52.7)                 |         |
| Poor, n (%)                   | 17 (23.3)                | 30 (40.5)                 |         |
| Recurrence                    |                          |                           | 0.982   |
| Yes, n (%)                    | 42 (72.4)                | 39 (72.2)                 |         |
| No, n (%)                     | 16 (27.6)                | 15 (27.8)                 |         |

One-way and repeated-measures analysis of variance (ANOVA) was performed in Table 1. The bold values indicate p < 0.05.

NEK2 inhibition activates an anti-pancreatic cancer immune response. To investigate the influence of NEK2 inhibition on pancreatic tumorigenesis and growth, pancreatic cancer cells both with and without pretreatment using a NEK2 inhibitor were injected s.c. into C57BL/6 and nude mice, respectively (Fig. 3a). Pretreatment with NEK2 inhibitor resulted in a lower incidence of tumors and postponed tumor occurrence (Fig. 3b), whereas a similar trend was not observed in nude mice (Fig. 3c, d). Furthermore, tumor-bearing C57BL/6 and nude mice were individually administered with NEK2 inhibitor and tumor growth was significantly suppressed in C57BL/6 mice (Fig. 3e–g) but not in nude mice (Fig. 3h–j). Moreover, the NEK2 inhibitor imposed no significant effect on the weight of mice compared with the control group (Fig. 3k, l). Additional immunohistochemical assays indicated a significant decrease in PD-L1 expression and increased CD8+ T cell infiltration in NEK2 inhibitor-treated tumors, suggesting that NEK2 inhibition may result in downregulation of PD-L1 and an enhancement of T cell function (Fig. 3m–o). In support of this, the specific NEK2 inhibitor significantly enhanced T cell-mediated tumor cell destruction in vitro (Supplementary Fig. 5c, d). Collectively, the results demonstrate that NEK2 inhibition suppressed the onset and development of pancreatic cancer, possibly associated with the decreased expression of PD-L1.

NEK2 positively correlates and interacts with PD-L1 in pancreatic cancer. Since PD-L1 is a well-recognized and quite important factor in the mediation of tumor immune resistance, we further investigated the regulatory effects of NEK2 on PD-L1 expression. The relationship between NEK2 and PD-L1 was first identified as co-occurrence at a genomic alteration level by mutual exclusivity analysis (Supplementary Fig. 6a). It was further confirmed to have a positive association in multiple assays, including, but not limited to, at the transcriptional level in the TCGA database (Supplementary Fig. 6b) and at the protein level in paired clinical tissue samples (Fig. 4a), in addition to tumor tissue microarrays (Fig. 4b–d). Moreover, a significant correlation was observed in KPC (KrasLSL-G12D; Trp53LSL-R172H, Pdx1-Cre) and KTC (KrasLSL-G12D/+; Tgfbrelox/wt, Ptf1a-Cre) autochthonous pancreatic tumors in a genetically engineered mouse model (GEMM) (Supplementary Fig. 6c–e). Expression levels of PD-L1 were significantly reduced in NEK2 KD tumors compared with WT control tumors in a xenograft mouse model (Supplementary Fig. 7a, b). Furthermore, an endogenous interaction between NEK2 and PD-L1 in multiple pancreatic cell lines was observed (Fig. 4f, g). Moreover, glutathione S-transferase (GST)-pull down assay showed that NEK2 bound to PD-L1 directly (Fig. 4h). Consistently, Duo-link assay demonstrated the real binding between NEK2 and PD-L1 in the cell (Fig. 4i). In further investigation of the localization of NEK2–PD-L1 interaction, no signals were detected with antibodies against both cytosolic and luminal proteins after trypsinization in the permeable fraction; instead, signals of the cytosolic domain of IRE1α were rapidly
reduced after trypsinization, whereas signals of NEK2 and HSP90B1 were maintained in the non-permeable fraction (Supplementary Fig. 8a). Taken together, the results indicate a positive correlation and direct interaction of NEK2 with PD-L1 inside the ER lumen in pancreatic cancer.

NEK2 inhibits ubiquitination-mediated proteasomal degradation of PD-L1. To further investigate the manner in which NEK2 regulates PD-L1, we measured the expression levels of PD-L1 in NEK2 inhibitor-treated, NEK2 KD, or overexpressed cells. NEK2 inhibition or depletion decreased PD-L1 expression in pancreatic cancer.
cancer cells (Fig. 5a, b), while the overexpression of NEK2 induced the upregulation of PD-L1 (Fig. 5c). Interestingly, downregulated PD-L1 expression caused by NEK2 depletion or inhibition could be restored by treatment with the proteasome inhibitor MG132, suggesting that NEK2 prevents proteasome-mediated degradation of PD-L1 (Fig. 5d, e). Indeed, PD-L1 in both NEK2 KD and inhibition groups exhibited a shorter half-life than control groups, a reduction that was also rescued by MG132 (Fig. 5f–i). Furthermore, increased levels of ubiquitination were observed in NEK2-deficient conditions (Fig. 5j, k). Collectively, these results suggest that NEK2 controls the stability of PD-L1 in a kinase-dependent manner. As demonstrated by Hung’s group, glycogen synthase kinase 3β (GSK3β) is an additional key protein that interacts with PD-L1 and induces phosphorylation-dependent proteasome degradation of PD-L1. Thus, the relationship between the role of NEK2 and GSK3β in regulating the expression level of PD-L1 was further explored. Interestingly, our results revealed that NEK2 inhibitor reduced the phosphorylation of GSK3β, but reversed the up-regulation of PD-L1 caused by the down-regulation of p-GSK3β, and affected the PD-L1–GSK3β interaction (Supplementary Fig. 9a–d). In contrast, the effect of the GSK3β inhibitor on NEK2 and PD-L1 expression was not very stable and significant in pancreatic cancer cells (Supplementary Fig. 9e, f). These results suggest that NEK2 plays a more important role than GSK3β in the regulation of PD-L1, at least in pancreatic cancer.

NEK2 maintains the stability of PD-L1 by phosphorylation at T193/T209. In addition to upregulation of PD-L1 expression, its glycosylation at the N192/N200/N219 residues was also stimulated by NEK2 overexpression (Supplementary Fig. 10a–c). The influence of glycosylation on PD-L1 stability can be regulated by kinase-mediated phosphorylation, suggesting NEK2 controls the protein level of PD-L1 in such a manner. Two evolutionarily conserved NEK phosphorylation-specific motifs (F/LXXS/T) were identified across multiple species, centering at the T194 and T210 residues (Mus musculus: T193 and T209) in the glycosylation-rich region (N192/N200/N219) (Fig. 6a). Specific antibodies designed to recognize PD-L1 phosphorylated at T194 and T210 (T193/T209 in the mouse) were specifically generated to detect potential modification of NEK2 on PD-L1 (Fig. 6b). A significant decrease in T193/T209 phosphorylation levels of PD-L1 was observed in the presence of the NEK2 inhibitor and in NEK2 KD cells (Fig. 6c). In vitro kinase assay demonstrated that NEK2 directly phosphorylated PD-L1 (Fig. 6d). To further investigate the influence of NEK2-mediated phosphorylation on PD-L1 expression levels, constitutively non-phosphorylated (T193/209A) and phosphorylated (T193/209D) mutants of PD-L1, in addition to a kinase-dead (K37R) mutant of NEK2, were individually constructed. The results demonstrated that the T193/209A mutation in PD-L1 reduced its expression but the T193/209D mutation increased its expression in pancreatic cancer cells, while the K37R mutation in NEK2 caused its loss of regulatory effect on the expression and phosphorylation of PD-L1 (Fig. 6e–g). Moreover, a shortened or extended half-life of PD-L1 was observed with T193/209A and T193/209D mutants, respectively (Supplementary Fig. 11a–d), while no effect is observed with the NEK2 K37R mutant on PD-L1 half-life (Supplementary Fig. 11e, f). Furthermore, increased and decreased ubiquitination of PD-L1 were observed in PD-L1 T193/209A and T193/209D mutants, respectively (Fig. 6h), while no influence was consistently observed with the NEK2 K37R mutant on PD-L1 ubiquitination (Fig. 6i). Taken together, the results demonstrate that NEK2 maintains PD-L1 stability through phosphorylation at the T193/T209 residues.

NEK2 inhibition sensitizes PD-L1-targeted pancreatic cancer immunotherapy. Since NEK2 regulates the stability of PD-L1, we hypothesized that inhibition of NEK2 may enhance the therapeutic efficacy of PD-L1-targeted drugs. Indeed, NEK2 inhibitor plus anti-PD-L1 antibody significantly improved the T cell-mediated cytotoxic effect in vitro compared with mono-NEK2 inhibitor or mono-anti-PD-L1 antibody (Supplementary Fig. 12a, b). In light of this, we additionally administrated NEK2 inhibitor and/or anti-PD-L1 antibody to KPC-bearing mice (Fig. 7a), after which both tumor volumes (Fig. 7b, c) and tumor weight (Fig. 7d) decreased significantly in mice that received the combined treatment. Additionally, the combination of NEK2 inhibitor and anti-PD-L1 antibody did not influence the weight of the mice (Fig. 7e). Additional flow cytometric analysis revealed a significant elevation in the quantity and activation of T cells infiltrated into the tumors in which both therapies were administered (Fig. 7f, g). Moreover, the combinatorial strategy was also evaluated in an orthotopic model (Supplementary Fig. 13a), where a similar outcome was observed (Supplementary Fig. 13b–d). Additionally, it was confirmed that the NEK2 inhibitor functionally inhibited NEK2 activity in mice (Supplementary Fig. 13e). Collectively, the results suggest that the NEK2 inhibitor enhanced the efficacy of PD-L1 blockade both in vitro and in vivo, alleviating immuno-resistance in pancreatic cancer.

Discussion

Increasing evidence suggests that conventional mediators of the cell cycle are also involved in cancer immune regulation. For instance, cyclin-dependent kinase 4, 5, and 6 (CDK4/5/6), three serine/threonine kinases that are conserved throughout eukaryotes, act as the fundamental driving forces for cell cycle progression and thus play an important role in the occurrence and development of multiple malignant tumors. Intriguingly, it has been reported that CDK4/6 inhibitors not only suppress tumor cell growth by inducing cell cycle arrest, they also trigger tumor immune evasion through inhibition of cullin 3–SPOP E3 ligase-mediated proteasomal degradation of PD-L1 that increases...
the abundance of the protein\textsuperscript{33}. Furthermore, combination therapy of CDK4/6 inhibitors and PD-1/PD-L1 blockade antibodies improve the efficacy of immunotherapy in multiple cancer types\textsuperscript{32–35}. CDK5 regulates the expression of PD-L1 via the interferon regulatory factors IRF2 and IRF2BP2 in medulloblastoma\textsuperscript{36}. As mentioned above, NEK2 is a multifunctional protein involved in non-immune and immune function including cell cycle regulation, microtubule stabilization, kinetochore attachment, spindle assembly checkpoint, phosphorylation of downstream proteins, and the maintenance of normal development and function of B cells, and has been associated with tumor progression and clinical prognosis in multiple cancers. In the present study, NEK2 has been identified as another critical regulatory factor in cancer immune resistance.
Fig. 3 NEK2 inhibition enhances the anti-pancreatic cancer immune response. a, c Schematic protocols of pancreatic cancer cells with or without pretreatment with a kinase-specific inhibitor of NEK2 (10 μM, 24 h) separately and s.c. injected into immunocompetent and immunodeficient mice (n = 7). b, d Tumor incidence in immunocompetent and immunodeficient mice was recorded at the times indicated. Tumors were more frequently treated using an NEK2 inhibitor (100 μg/mouse, 2 weeks). e, f Schematic protocol of the treatment schedule and tumor growth curve in immunocompetent mice (n = 5). Tumors were measured at the time points indicated and removed at the endpoint. (h, i) Schematic protocol of the treatment schedule and tumor growth curve in immunodeficient mice (n = 5). Tumors were measured at specified time points and dissected at the endpoint. Tumor and mouse weights in immunocompetent (g, k) (n = 5) and immunodeficient mice (j, l) (n = 5) as reported at the endpoint. m–o Representative images and further quantification of tumor-infiltrating lymphocytes. Scale bars: 100×: 50 μm; 200×: 100 μm. Kaplan-Meier method and a Gehan-Breslow–Wilcoxon test are indicated in b and d. Results represent means ± SD of one representative experiment in f, g, i, j, k, l, m, and n. All data are representative of three independently performed experiments. *P < 0.05, **P < 0.01, ***P < 0.001 using a two-tailed t-test; ns: not significant.

Overall, these findings provide a theoretical basis for the development of an anti-cancer treatment strategy combining cell cycle-related kinase inhibitors with PD-1/PD-L1-targeted immunotherapy.

PD-L1 is expressed in a variety of cells, especially cancer cells and macrophages, and is critical for tumors to escape immune surveillance. To date, PD-1/PD-L1 blockade has become the most successful strategy in cancer immunotherapy against solid tumors. However, the overall response rate for PD-1 or PD-L1 blockade rarely exceeds 40%. One reason that accounts for this phenomenon is the PTM of PD-L1, which potentially impacts the therapeutic efficacy of PD-L1 blockade. The mechanism of PD-L1 PTM has been extensively investigated and multiple regulators affecting PD-L1 stability via PTMs have been identified, providing methods to improve the efficacy of PD-L1 blockade.

For instance, EGF signaling induces PD-L1 glycosylation, while Gefitinib (an EGFR inhibitor) sensitizes PD-L1 blockade therapy in a syngeneic mouse model. Based on these findings, the present study further demonstrated that NEK2 is a critical regulator of PD-L1 phosphorylation, and is involved in tumorigenesis and the progression of pancreatic cancer. NEK2 directly interacts with and phosphorylates PD-L1 to maintain its stability. The combination of a NEK2 inhibitor and anti-PD-L1 antibody synergistically suppresses tumor growth both in vivo and in vitro, thus representing the potential for sensitizing PD-L1-targeted therapy in pancreatic cancer.

Given the considerable influence of the tumor immune microenvironment on immune checkpoint blockade (ICB) therapy, we investigated the prognostic roles of NEK2 in pancreatic cancers with different immune statuses. NEK2 was significantly correlated with the prognosis of patients of immunologically “hot” status, while no significant correlation was observed in immunologically “cold” PDAC. The presence of tumor-killing T cells demonstrates the capability of the immune system to destroy tumors and its sensitivity to ICB.

PDAC is characterized by substantial immunological heterogeneity and differential levels of T cell infiltration. “Hot” tumors typically respond well to checkpoint treatments. Hence, NEK2 may be considered a therapeutic biomarker for ICB, and intervention with NEK2 may enhance the efficacy of PD-L1 blockade in “hot” pancreatic tumors. In addition to T cells, a great number of immunosuppressive populations are present in PDAC, also greatly influencing the efficacy of checkpoint therapy. Interestingly, the expression of both NEK2 and PD-L1 was also observed in macrophages, DCS, and MDSCs in addition to tumor cells. In light of this, we speculated that NEK2 may also regulate the expression of PD-L1 in these suppressive populations. TMB is a measure of the number of somatic mutations in tumors and can be treated as a biomarker that reflects the potential therapeutic efficacy of immunotherapy, allowing the identification of patients who may benefit. A high mutation load correlates with increased numbers of neoantigens, leading to T cell immunoreactivity and sensitivity to ICB treatments.

Methodology

Antibodies. Antibodies were obtained from the following sources: anti-NEK2 (sc-33601, Santa Cruz, 1:200; 24171-1-AP, Proteintech Group, 1:2000; 610594, BD biosciences, 1:100), anti-Phospho-NEK2 (Ser170) (PA5-10332, Invitrogen, 1:1000), anti-PD-L1 (13684, Cell Signaling Technology, 1:100; ab213480, Abcam, 1:1000; ab250921, Abcam, 1:1000; 662481-1-Ig, Proteintech Group, 1:2000; 14:5983-82, Thermo Scientific, 1:100), anti-Ubiquitin (3933, Cell Signaling Technology, 1:1000), anti-Cd8a (98941, Cell Signaling Technology, 1:400), anti-GSK-3β (2624, Cell Signaling Technology, 1:1000), anti-IRE1α (3294, Cell Signaling Technology, 1:1000), anti-HSP90B1 (NBP2-42379, Novus Biologicals, 1:500), anti-EGFR (4267T, Cell Signaling Technology, 1:1000), anti-GST (2624, Cell Signaling Technology, 1:1000), anti-f-actin (Ab8226, Abcam, 1:2000), anti-GAPDH (AF5009, Beyotime, 1:1000), anti-α-tubulin (AF0081, Beyotime, 1:2000), anti-Flag (2368, Cell Signaling Technology, 1:1000), anti-GFP (2958, Cell Signaling Technology, 1:1000), rabbit IgG isotype control (8726, Cell Signaling Technology, 1:100), mouse IgG isotype control (sc-2025, Santa Cruz, 1:200), HRP goat anti-
Fig. 4 NEK2 positively correlates and interacts with PD-L1 in pancreatic cancer. 

(a) Western blot analysis of NEK2 and PD-L1 in clinical pancreatic tissue samples from patients (n = 20) (N: Normal pancreatic tissue; T: Pancreatic tumor tissue).

(b)–(d) Representative images and statistical results of IHC staining of NEK2 and PD-L1 in a tissue microarray (n = 156).

(e)–(g) Cell lysates from SW1990, KPC, and CFPAC-1 separately analyzed by IP and Western blotting using the antibodies indicated. Representative image is shown n = 3 independent experiments.

(h) GST-pull down assay of NEK2-His and GST-PD-L1 protein. Representative image is shown n = 3 independent experiments.

(i) Representative images of individual immunofluorescence staining of NEK2 and PD-L1 interaction in KPC cells by Duolink assay. The red dots (NEK2/PD-L1 interaction) indicate their interaction. Representative image is shown n = 3 independent experiments. The Spearman correlations and p-values by Spearman’s test are indicated in (d).
rabbit IgG (A0208, Beyotime, 1:5000 for WB, 1:100 for IHC), HRP goat anti-mouse IgG (A0216, Beyotime, 1:5000 for WB, 1:100 for IHC), goat anti-rabbit IgG (GTX77061, GeneTex, 1:2000), goat anti-mouse IgG (GTX26708, GeneTex, 1:2000), FITC anti-mouse CD3 (100203, Biolegend, 1:200), APC/Cy7 anti-mouse CD3 (100329, Biolegend, 1:200), Brilliant Violet 785 anti-mouse CD8a (100722, Biolegend, 1:200), Brilliant Violet 605 anti-mouse CD8a (100744, Biolegend, 1:200), FITC anti-mouse IFN-γ (505806, Biolegend, 1:200), Brilliant Violet 421 anti-human/mouse Granzyme B (329714, Biolegend, 1:200), PE rat IgG2b, κ isotype control

Rabbit IgG (A0208, Beyotime, 1:5000 for WB, 1:100 for IHC), HRP goat anti-mouse IgG (A0216, Beyotime, 1:5000 for WB, 1:100 for IHC), goat anti-rabbit IgG (GTX77061, GeneTex, 1:2000), goat anti-mouse IgG (GTX26708, GeneTex, 1:2000), FITC anti-mouse CD3 (100203, Biolegend, 1:200), APC/Cy7 anti-mouse CD3 (100329, Biolegend, 1:200), Brilliant Violet 785 anti-mouse CD8a (100722, Biolegend, 1:200), Brilliant Violet 605 anti-mouse CD8a (100744, Biolegend, 1:200), FITC anti-mouse IFN-γ (505806, Biolegend, 1:200), Brilliant Violet 421 anti-human/mouse Granzyme B (329714, Biolegend, 1:200), PE rat IgG2b, κ isotype control
Fig. 5 NEK2 inhibits ubiquitination-mediated proteasomal degradation of PD-L1. a, b Western blot analysis and flow cytometry of PD-L1 expression in pancreatic cancer cell lines after treatment with NEK2 inhibitor (10 μM, 24 h) and NEK2 knockdown. Representative image is shown n = 3 independent experiments. c LC-MS proteomics quantitative analysis of pancreatic cancer cell lines overexpressing NEK2. d Western blot analysis of PD-L1 expression in KPC cells treated with NEK2 inhibitor (10 μM, 24 h). Representative image is shown n = 3 independent experiments. e Western blot analysis of PD-L1 expression in WT and NEK2 KD KPC cells treated with MG132 (100 μM, 24 h). Representative image is shown n = 3 independent experiments. f Stability analysis of PD-L1 in KPC cells treated with NEK2 inhibitor (10 μM, 24 h) after treatment with cycloheximide (CHX) (20 μg/mL). Representative image is shown n = 3 independent experiments. g Stability analysis of PD-L1 in WT and NEK2 KD KPC cells treated with MG132 (50 μM, 24 h). Representative image is shown n = 3 independent experiments. h Statistical analysis of three independent experiments is displayed. The intensity of PD-L1 protein expression was quantified using a densitometer. i Ubiquitination assay of PD-L1 in KPC cells treated with NEK2 inhibitor (10 μM, 24 h), subjected to anti-PD-L1 IP and anti-ubiquitin Western blot analysis after treatment with MG132 (50 μM, 24 h). j Ubiquitination assay of PD-L1 in WT and NEK2 KD KPC cells treated with MG132. Results represent means ± SD of one representative experiment in h and i. All data are representative of three independently performed experiments. *P < 0.05, **P < 0.01, ***P < 0.001 using a two-tailed t-test; ns: not significant.
**T cell-mediated tumor cell destruction assay.** CD8⁺ T cells were isolated from the mouse spleen using a CD8⁺ T cell isolation kit, an LS Column, and a MidiMACS™ Separator. Isolated CD8⁺ T cells were fluorescently stained using CD8-PE and CD3-FITC then analyzed using flow cytometry to confirm their phenotype. KPCs both untreated and treated with NEK2 inhibitor (10 μM) for 24 h were incubated for 48 h with activated CD8⁺ T cells. KPC-NEK2 KD cells and KPC-WT cells were incubated overnight and allowed to adhere to the culture plates, then treated with isolated activated CD8⁺ T cells and incubated for 48 h. KPC cells were incubated overnight to allow adhesion to the culture plates and then treated with isolated activated CD8⁺ T cells with NEK2 inhibitor (10 μM) and anti-PD-L1 (200 μg), either individually or in combination, then incubated for 48 h. The ratio of tumor cells to activated CD8⁺ T cells was 1:4. T cells and cell debris were removed by washing three times with PBS. The remaining living cancer cells were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet, and quantified using a spectrophotometer at OD 570 nm.

**Immunoblotting and immunoprecipitation.** For immunoblotting, cells were lysed using RIPA lysis buffer (P0013B Beyotime Biotechnology) containing 5% 2-mercaptoethanol (R) and 0.05% Tween-20. For immunoprecipitation, cells were lysed using RIPA lysis buffer (P0013B Beyotime Biotechnology) containing 5% 2-mercaptoethanol and 0.05% Tween-20. The lysates were incubated with the primary antibody overnight at 4°C and then incubated with protein-G agarose beads for 2 h at 4°C. The beads were washed three times with PBS and then boiled in SDS-PAGE sample buffer for 5 min. The proteins were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk in TBS-T for 1 h, then incubated with the primary antibody overnight at 4°C, and then incubated with the secondary antibody for 1 h. The blots were developed using an ECL chemiluminescence kit.
Fig. 6 NEK2 phosphorylates PD-L1 at T194/T210 to maintain protein stability. a Schematic diagram of the NEK-binding motif (F/LXXS/T) in the glycosylation-rich region and amino acid sequences around the potential binding sites of PD-L1 were aligned in evolutionarily divergent species. The F/LXXS/T motifs are highlighted in blue. b Generation of site-specific antibodies against T194 and T210 (Mus musculus: T193 and T209)-phosphorylated PD-L1. c Western blot analysis of T193 and T209-phosphorylated PD-L1 in KPC cells with NEK2 inhibitor (10 μM, 24 h) and NEK2 KD KPC. Representative image is shown = n = 3 independent experiments. d In vitro kinase assay and western blot analysis of pT193-PD-L1 and pT209-PD-L1 expression of recombinant PD-L1 WT and NEK2 (active) protein. Representative image is shown, n = 3 independent experiments. e, f Western blot analysis of PD-L1 expression in flag-PD-L1 WT and T193/209A or T193/209D-transfected KPC cells with or without MG132 treatment. Representative image is shown = n = 3 independent experiments. g Western blot analysis of pT193-PD-L1, pT209-PD-L1, and PD-L1 expression in WT and K37R transfected NEK2 KD KPC cells. Representative image is shown = n = 3 independent experiments. h Ubiquitination assay of PD-L1 in Flag-PD-L1 WT and T193/209A or T193/209D-transfected KPC cells, subjected to anti-PD-L1 IP and anti-ubiquitin Western blot analysis after treatment with MG132 (50 μM, 24 h). Representative image is shown = n = 3 independent experiments. i Ubiquitination assay of PD-L1 in WT and K37R transfected NEK2 KD-KPC cells subjected to PD-L1 IP and Western blot analysis with anti-ubiquitin after treatment with MG132 (30 μM, 24 h). Representative image is shown = n = 3 independent experiments.

Immunofluorescence staining. To show the binding between NEK2 and PD-L1, pancreatic cancer cells were subjected to Duo-link assay (catalog DUO92101, Sigma-Aldrich) with anti-NEK2 (PAS-312359, Thermo Scientific) and anti-PD-L1 (14-5983-82, Thermo Scientific) as primary antibodies, according to the manufacturer’s instructions. Fluorescent images of the cells were observed under a TCS SPX confocal microscope (Leica).

Trypsinization of ER microsomal fractions. ER fraction was collected from pancreatic cancer cells by ER enrichment kit (NBP2-29482, Novus Biologicals). After pretreatment with or without 1%, Triton X-100 for 3 min, the solution (0.625 g/L trypsin + 0.05 g/L EDTA in PBS) was added to ER fraction. Samples were incubated for the indicated time. After trypsinization, samples were analyzed by Western blotting with primary antibodies against IRE1α (Proteintech), TRP53 (Bioss), and GRP78 (Proteintech) as secondary antibodies, according to the manufacturer’s instructions.

In vitro kinase assay. Recombinant PD-L1 WT (10084-H08H, sinobiological) were incubated with activated NEK2 (active) (14-545, Sigma-Aldrich) and 200 μM ATP (9040S, Cell Signaling Technology) in a kinase buffer (9802S, Cell Signaling Technology) at 30 °C for 30 min. The kinase reaction was stopped by the addition of SDS sample and boiling and subsequently subjected to Western blotting.

Flow cytometric analysis and CTL profile analysis of mouse tumors. Samples were mechanically dissociated into small fragments using scissors and scalpels, placed in DMEM supplemented with 2% FBS, collagenase IV (1 mg/ml) (17104019, Thermo Fisher Scientific), Dnase (10 μg/ml) (D5025, Sigma-Aldrich), Dispase (0.6 mg/ml) (17105041, Gibco), and Collagenase (3 ml) (21115, Sigma-Aldrich), then incubated at 37 °C while shaking at 200 rpm for 40–60 min. Digestion was terminated by the addition of RPMI containing 10% FBS, after which the dissociated tissues were filtered through a 40-μm cell strainer (80771-1, Thermo Fisher Scientific) then washed in PBS. The cells were resuspended in 36% Percoll solution (GE Healthcare) containing 4% 10× PBS and 60% serum-free DMEM then separated using density gradient centrifugation to remove non-immune cells. The immune cells were stimulated with a leukocyte activation cocktail (5505B, BD Biosciences), incubated at 37 °C for 4–6 h in accordance with the manufacturer’s instructions, and stained with a LIVE/DEAD Fixable Violet dead cell staining kit on ice for 30 min in the dark. The cells were then washed with PBS, blocked using human TruStain FcX™ (anti-mouse CD16/32 antibody) then stained on ice for the cell surface epitopes CD45, CD3, and CD8 in PBS supplemented with 2% FBS for 20 min in the dark. The cells were washed again with PBS, fixed then permeabilized using a fixation/permeabilization solution kit (555028, BD Biosciences). The cells were also stained for intracellular Granulysin B, Perforin, TNF-α, and IFN-γ in permeabilization solution (555028, BD Biosciences). All samples were analyzed by flow cytometry using a Beckman CytoFLEX LX and data was analyzed by FlowJo software.

Profile analysis of immunosuppressive population in PDAC tumors. Patient samples were dissociated and digested, as described above. The cells were stained using a LIVE/DEAD Fixable Violet dead cell stain kit and incubated for 30 min on ice in the dark. The cells were then washed with PBS, blocked using human TruStain FcX™ (Fc receptor blocking solution), stained with antibodies for the cell surface epitopes CD45, C11b, CD11c, CD15, CD68, and HLA-DR dissolved in PBS.
Fig. 7 NEK2 inhibition sensitizes PD-L1-targeted pancreatic cancer immunotherapy. a Schematic protocol of the combination of anti-PD-L1 antibody and NEK2 inhibitor therapy. b Tumor growth curve of mice treated with anti-PD-L1 antibody (200 μg/mouse), NEK2 inhibitor (100 μg/mouse), or their combination (n = 5). c, d Representative images displaying tumors harvested from mice bearing KPC cells treated with anti-PD-L1 antibody, NEK2 inhibitor, or their combination (n = 5). f, g Flow cytometric analysis and statistical results of lymphocytes that have infiltrated the tumors (n = 5). Results are presented as means ± SD from one representative experiment in b, d, e, and g. All data are representative of three independently performed experiments. *P < 0.05, **P < 0.01, ***P < 0.001 using a two-tailed t-test; ns: not significant.
containing 2% FBS, then incubated for 30 min on ice in the dark. The cells were washed again with PBS, fixed and permeabilized using fixation/permeabilization solution kit (555028, BD Biosciences), then stained for intracellular NEK2 (610594, BD Biosciences) in permeabilization solution (555028, BD Biosciences). The cells were washed again with permeabilization solution and stained for rat anti-NEK2 antibody (55083, BD Biosciences). All samples were analyzed by flow cytometry using a Beckman CytoFLEX SX. All flow cytometry data were analyzed using FlowJo software.

Antibody generation and detection of anti-phospho-T194/T210-PD-L1 antibodies. To generate custom antibodies against PD-L1, a 10–16-week process comprising four phases was conducted, as follows. Phase I included the design and synthesis of the T194/T210-PD-L1 peptide (1–3 weeks). Conjugated peptides were then used to immunize rabbits through 4–6 rounds of immunization in phase II (8–12 weeks). Pre-immune (5 mL) and immunized blood (100–120 mL) were collected and immunized blood precipitated using ammonium persulfate, and antibodies purified by procedures that included antigen affinity and special affinity purification. The resultant samples were suspended in PBS containing 0.03% sodium azide (phase III). The specificity of each purified antibody was verified in phase IV.

LC-MS/MS analysis. Mass spectrometry analysis was performed by PTM-Biolabs Co., Ltd. (Hangzhou, China). MS/MS data were processed using a Maxquant search engine (v1.5.2.8). Tandem mass spectra were searched against the human uniprot database concatenated with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme, allowing up to 4 missing cleavages. Mass tolerance for fragment ions was set to 0.02 Da. Cysteine carbamidomethylation was specified as a fixed modification and acetylation and oxidation of Met were specified as variable modifications. FDR was adjusted to <1% with a minimum score for modified peptides set to >40. Proteins were classified using GO annotation into three categories: biological process, cellular compartment, and molecular function. For each category, a two-tailed Fisher’s exact test was used to test for enrichment of differentially expressed proteins against all identified proteins. Corrected p-values <0.05 were considered statistically significant.

Quantification and statistical analysis. Statistical analysis was performed using SPSS (V20, IBM Corp., Armonk, NY, USA) and Prism software (GraphPad Inc, version 7.0). Data for all experiments from at least three biological replicates are presented as means ± SD. The significance of the difference between the two groups was evaluated using a Student’s t-test, while one-way and repeated-measures analysis of variance (ANOVA) was used to compare multiple groups. Spearman’s rank correlation was performed to analyze the correlation between variables. The overall difference in data at the endpoint was assessed using a Student’s t-test to evaluate tumor growth. The difference in survival curves was analyzed using the Kaplan–Meier method and a Gehan–Breslow–Wilcoxon test. P values < 0.05 were considered statistically significant.

Data availability

Figure 1a, e–h, Supplementary Figs. 1a, b, 2a, b, 4a, b, 6a and b were generated from publicly available databases from TCGA, ICGC, and GTEx by GEPIA2 (http://gepia2.cancer-pku.cn), TISIDB (http://cistrome.shangai.cn/TISIDB), Kaplan–Meier Plotter (http://kmplot.com/analysis), and CBioPortal for cancer genomics (http://www.cbioportal.org). The proteomic data have been deposited in PRIDE database under the accession code PXD026877. The remaining data are available within the Article, Supplementary Information or Source Data file. Source data are provided with this paper.

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**Fig. 8 Predicted model of the NEK2-PD-L1 signaling pathway in pancreatic cancer.** A schematic model is proposed to illustrate how PD-L1 protein stability is regulated by NEK2 in pancreatic cancer. NEK2 positively regulates and interacts with PD-L1 largely through PD-L1 phosphorylation at the T194/T210 residue in ER of pancreatic cancer. Therefore, treatment with NEK2 inhibitor unexpectedly suppressed PD-L1 protein expression, largely by inhibition of PD-L1 phosphorylation that promotes its degradation.
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Author contributions

T.-B.L., X.-L.B., and X.H. supervised the study. X.H. conceived the project and designed the experiments; X.-Z.Z. performed the majority of experiments and acquired the data; X.H., T.-B.L., and X.-L.B. conducted data analyses and interpreted the results; X.-L.B.; 81871925 and 82071867 to X.B.; 31970696 and 81502975 to X.H.), Key Research.

Competing interests

The authors declare no competing interests.
Additional information

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