LIN28/let-7/PD-L1 Pathway as a Target for Cancer Immunotherapy

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Abstract

The immunocheckpoint protein PD-1/PD-L1 is considered a promising target for cancer immunotherapeutics. However, the objective response rate using antibodies that block the interaction between PD-1 and PD-L1 was less than 40%, and the mechanism underlying regulation of PD-1/PD-L1 expression is poorly understood. In this study, we identified the miRNA let-7 that posttranscriptionally suppresses PD-L1 expression. LIN28, an RNA binding protein upregulated in most cancer cells, inhibits the biogenesis of let-7, thus promoting PD-L1 expression. Therefore, inhibition of LIN28 may be a strategy to prevent immune evasion of cancer cells. We found that treatment with a LIN28 inhibitor, the small compound C1632, increases let-7 and suppresses PD-L1 expression, leading to reactivation of antitumor immunity in vitro and in vivo. In addition, C1632 also displayed the capacity to inhibit cancer cell proliferation and tumor growth in mice. Altogether, these findings identified LIN28/let-7 as a target for PD-L1–mediated immunotherapeutics and reveal the potential of C1632 and its derivatives as promising oncotherapeutic agents.

Introduction

Programmed death ligand-1 (PD-L1), also known as CD274 or B7-H1, is a type I transmembrane protein that interacts with the T-cell inhibitory receptor programmed cell death protein-1 (PD-1). The PD-1/PD-L1 complex on the cell surface promotes T-cell tolerance and minimizes autoimmune-mediated inflammation under normal physiologic conditions (1). Thus, PD-L1 is considered to be an immune-checkpoint protein. However, PD-L1 is often overexpressed in cancer cells, most likely as a mechanism to escape immune surveillance, avoid recognition of tumor-specific antigens by T cells, and promote tumor progression. High expression of PD-L1 is associated with more aggressive and malignant tumor subtypes, higher risk of cancer mortality, and poor patient prognosis (2). Clinical trials that tested the ability of monoclonal antibodies against PD-L1 to improve the outcome of immunotherapy for kidney, lung, and melanoma skin cancer had promising results (3–6), despite a low objective response rate (less than 40%). This could reflect individual variation in expression of PD-L1 and the complexity of the tumor microenvironment (7, 8). Specifically, poor responders may express low PD-L1 or demonstrate less frequent infiltration of tumor tissue by antibodies or T cells. Thus, there is great need for new therapeutic modalities and a better understanding of how expression of PD-L1 is regulated.

LIN28 is an RNA binding protein first discovered in Caenorhabditis elegans (9). In mammals, the LIN28 family includes LIN28A and LIN28B, two protein isoforms that share similar structure and function (10). LIN28 regulates many biological processes, including stem cell differentiation and cell proliferation (11, 12). LIN28 is frequently dysregulated in tumor cells, and upregulation of LIN28 correlates with advanced disease and poor prognosis for many cancer subtypes (13). It is well established that LIN28A/LIN28B interferes with conversion of pre–let-7 transcripts to mature let-7 miRNAs, thereby suppressing let-7–mediated downstream effects (14, 15). The let-7 family includes 12 miRNA members, which are thought to play important roles as tumor suppressors (16). Let-7 binds to the 3′-UTRs of key oncogenes, including RAS and MYC, and inhibits their expression (17, 18). In addition, let-7 also suppresses cancer cell proliferation by inhibiting expression of cell-cycle regulators such as E2F2, CDK6, and CCND2 (19, 20). Therefore, let-7 is frequently downregulated in human cancers, and reduced expression of let-7 family members is highly associated with poor prognosis for cancer patients (21–23).

Here, we investigate the possibility that dysregulation of LIN28/let-7 plays a role in tumor cell–driven evasion of immune surveillance. We demonstrate that let-7 interacts with the 3′-UTR and suppresses expression of PD-L1 in multiple human cancer cell lines, and this mechanism is in tumour blocked by LIN28. However, an inhibitor of LIN28, C1632, restores let-7–mediated downregulation of PD-L1, inhibits tumor cell growth, and prevents...
evasion of immune-mediated tumor surveillance in vitro and in vivo. These results reveal a targetable pathway that regulates immune surveillance and cancer cell proliferation simultaneously, and may provide a mechanism for improving the efficacy of antitumor immunotherapies.

Materials and Methods

Cell culture and transfection

Human 293T, Hela, MCF-7, U2OS, A549, MNNG/HOS, SKLI-1, and mouse TUBO and 4T1 cells were obtained from Chinese Typical Culture Center from 2014 to 2015. MG63, SAOS2, and THP-1 cells were kindly provided by Zhou Songyang's lab at 2016. All the cells were verified by standardized short tandem repeat analysis. In our lab, each new aliquot was resuscitated, and then new seed stocks were thawed. Mycoplasma was regularly examined during cell culturing, and no contamination occurred during this study. Cells were grown in Dulbecco's modified Eagles medium (Life Technologies) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C in 5% CO2. For gene expression, cells were transiently transfected with plasmids using lipofectamine 3000 (Life Technologies). For knockdown experiments, siRNA or negative control (siRNA with scrambled sequence; GenePharma) were transfected into cells using RNAi Max (Life Technologies) according to the manufacturer's protocol.

Plasmids

Let-7 sensor plasmid psiCHECK2-let-7 4 x (#20930) and pBABE-hLin28B (#26358) were purchased from Addgene. The Lin28A gene was obtained by amplification of its cDNA from human 293T cells. The PCR product was then inserted into pCDH-CMV-MCS-EF1-copGFP-T2A-Puro vector (cat. #CD511B-1, SBI) using NovoRec PCR Cloning Kit (Novoprotein Scientific Inc.). Let-7 expression vectors were kindly provided by Songshan Jiang's lab (24). To deplete PD-L1 in TUBO mouse cells, lentivirus vectors expressing shRNA were generated: pLVX-shRNA2-T2A-Puro and pBABE-hLin28B (#26358) were purchased from Addgene, respectively; shRNA sequence: GAGGTAATTTGGATA-TCCTTGTTCCAGATTACCTC. Transfection and assays were performed in triplicate. Assays were performed using the dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions. Transfection and assays were performed in triplicate.

Bioinformatic analysis

RegRNA software (online software provided by National Chiao Tung University, Taiwan, China. http://regrna.mbc.nctu.edu.tw/html/prediction.html) was used to predict binding sites for let-7 in PD-L1 mRNA.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted using RNAiso Plus (Takara) according to the manufacturer's instructions. cDNA was synthesized with PrimeScript II first-strand cDNA Synthesis Kit (Takara), followed by amplification with RealStar Power SYBR Mixure (GenStar). miRNAs were quantified using the stem-loop method, with U6 snRNA as internal control. Specific primer sequences were designed as described previously (25). qPCR was performed with a LightCycler 480 Real-Time PCR system (Roche). Data were analyzed using the comparative Ct (2-ΔΔCt) method (26). All experiments were performed in triplicate. qPCR primers for mRNA detection were as follows: GAPDH-forward 5'-AGTGGTCATGAGTCCTTCCACGATA-3', LIN28A-forward 5' (-ATATTCCAGTCGATGTATTTG-3); LIN28B-forward 5'-CCCGTCATGAGACACC-3', LIN28B-reverse 5'-TGCTGTCATGTCATTGATG-3'; PD-L1-forward 5'-ACC TACTGCGATTTGGCAG-3', PD-L1-reverse primer 5'-TCCCTCATTCCC AATAGACA-3'.

Dual-luciferase reporter assays

293T cells (2 x 10^4) were plated in 100 μl medium in 96-well plates. The cells were transfected with 100 ng reporter plasmid DNA and 300 ng miRNA expression plasmid DNA using PEI (Polyscience Inc.) and incubated for 48 hours. Transfected cells were harvested and assayed using the dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions. Transfection and assays were performed in triplicate.

LIN28 inhibitor synthesis

As shown in Scheme 1, CL285032 (N-methyl-N-[3-(3-methyl-1,2,4] triazolo[4,3-b]pyridazin-6-yl)phenyl]acetamide) was synthesized according to a literature-reported procedure (27). Initial raw materials 1 (N-(3-Acetylphenyl)-N-methylacetamide), 2 (3-methyl-4H-1,2,4-triazol-4-amine hydrochloride), and 4 (Bredereck reagent) were purchased from J&K Chemical Company. CL285032 was obtained by flash chromatography using MeOH/CH2Cl2 (0-15%) as a light-yellow solid. 1H NMR (CDCl3, d, ppm): 8.42 (d, J = 9.6 Hz, 1H), 8.11 (br s, 2H), 7.98 (d, J = 9.6 Hz, 1H), 7.66 (t, J = 7.8 Hz, 1H), 7.57 (d, J = 6.8 Hz, 1H), 5.24 (s, 3H), 2.78 (s, 3H), 1.85 (s, 3H). LC-MS (ESI): 

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\text{Scheme 1.} \quad \text{The synthesis of CL285032 (27).}
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Drug treatment

Cancer cells (1 × 10^5) were seeded in 6-well plates. C1632 was added to the culture medium at the indicated dose and incubated for 3 days. IFNγ was added to a final concentration of 10 ng/mL 24 hours before cell harvest, after which population doubling was calculated, and surface PD-L1 protein was analyzed by flow cytometry mean fluorescence intensity (MFI).

Cell viability assay

Cancer cells (5 × 10^3) were seeded in 96-well plates. DMSO or C1632 was added to the culture medium at the indicated dose and incubated for 3 days. Cell viability was measured with a kit (CCK-8) provided by Biotool.

Apoptosis assay

Apoptosis was determined by flow cytometry and immunoblot assay. Cells were infected with the Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech) and quantified by flow cytometry according to the manufacturer's instructions. For immunoblot, blots were probed with a primary antibody against Caspase-3 (Cell Signaling Technology). β-Actin (Proteintech) was used as a control.

Tumor-infiltrating lymphocyte analysis and flow cytometry

Twenty-four hours after the last injection of C1632, mice were anesthetized, and tumors were excised. Freshly isolated primary tumor tissue was digested in collagenase (Sigma) and DNase (Sigma) at 37°C for 1 hour. Lymphocytes were enriched on a Ficoll (Solarbio) gradient for flow cytometry analysis. T cells were stained using CD3-FITC (eBioscience), CD8-PE (eBioscience), CD69-APC (eBioscience), and CD279-PE (eBioscience). Tumor cells were isolated and analyzed by flow cytometry on a BD FACSariaII cytometer. Cells were stained with CD45-APC (eBioscience) to distinguish immune cells from tumor cells. Rat IgG2b-PE (eBioscience) antibodies were used as isotype controls. Data were analyzed using FlowJo V7.6 (Tree Star).

Determination of IFNγ and TNFα in serum samples

Twenty-four hours after the last injection of C1632, whole blood samples were collected as previously described (28). Serum was isolated, and relative concentrations of IFNγ and TNFα were measured by an ELISA kit following the protocol provided by the manufacturer (Multiscience).

Animal treatment protocol

Wild-type BALB/c mice and BALB/c nude mice (4-6-week-old, female) were purchased from the Laboratory Animal Center of Sun Yat-sen University (SYSU). Experiments were conducted under guidelines approved by the Institutional Animal Care and Use Committee of SYSU. TUBO- or PD-L1-depleted TUBO cells (5 × 10^5) in 50 µL PBS were injected into the mammary fat pad under #3 mammary gland in both wild-type and immune-deficient BALB/c mice. Tumor-bearing mice were randomly divided into two groups and intravenously treated with PBS or a LIN28 inhibitor at a dose of 40 mg/kg for 12 days (6 injections in total). Tumor growth was measured every 2 days with a digital caliper, and the formula V = length × width^2/2 was used to calculate tumor volume.

Statistical analysis

Statistical analyses were performed with GraphPad (GraphPad Prism) using a two-tailed Student t test. Values are presented as mean ± standard deviation (SD). P values < 0.05 were considered statistically significant (**, P < 0.01; *** P < 0.001). In RNA-seq data analysis, the Pearson correlation coefficient, r, and P values were calculated using GraphPad. P < 0.05 was considered to be statistically significant.

Results

Let-7 binds to the coding region and 3′-UTR of PD-L1 and suppresses translation

To test the idea that let-7 might regulate expression of PD-L1, transcriptome data from 1,189 breast carcinoma tissue biopsies were obtained from TCGA database and analyzed. The results revealed a moderate but statistically significant negative correlation between the expression of PD-L1 and let-7a (r = −0.083, P < 0.01) or let-7e (r = −0.118, P < 0.001; Fig. 1A). Similar analysis of transcriptome data from 1,045 lungs (Supplementary Fig. S1A) and 309 cervical carcinoma tissue biopsies (Supplementary Fig. S1B) in TCGA database also demonstrated statistically significant negative correlations between abundance of let-7 and PD-L1 transcripts. These data suggest that let-7 might negatively regulate expression of PD-L1.

Analysis of the PD-L1 mRNA using RegRNA revealed the presence of three putative let-7 binding sites, one in the PD-L1 coding region (target site 1) and two in the 3′-UTR (target sites 2 and 3; Fig. 1B). These predicted let-7 binding sites were cloned into a dual-luciferase reporter construct and assayed for ability to interact with let-7 and alter reporter gene activity. Let-7 significantly downregulated the activity of all three luciferase reporter gene constructs and that the strongest effects were observed when let-7a, let-7c, and let-7e bound to target site 1 (Fig. 1C). The specificity and functional effect of the binding of let-7 to putative target sites in PD-L1 was further tested by mutating three let-7 binding sites in PD-L1 and comparing the expression of wild-type (WT) and mutant PD-L1 in the absence (EV) and presence of let-7a, c, or e. Let-7a, c, or e suppressed expression of WT PD-L1 by approximately 30%, but did not suppress expression of PD-L1 with mutant variants of let-7 target sites (Fig. 1D). This confirms that let-7 negatively regulates expression of PD-L1, and this effect is mediated by let-7 binding to specific sequences in PD-L1.

Let-7 suppresses PD-L1 expression posttranscriptionally in human cancer cells

The following experiments investigated let-7 regulation of PD-L1 expression in MCF-7, U2OS, and Hela cancer cells using a let-7 "sponge" as a means to inhibit endogenous let-7 in these cells. The "sponge" has multiple RNA sequences complementary to the heptameric seeds of let-7 members (29). Here, the let-7 sponge was cotransfected into 293T cells along with a luciferase reporter plasmid carrying the let-7 complementary sequence fused to the luciferase gene (30). In cells expressing the let-7 sponge, luciferase activity was 155% higher (2.53-fold) than control cells expressing GFP (Supplementary Fig. S2A), confirming the specificity and efficiency of let-7 sponge.
Cellular PD-L1 protein increased by 1.5-fold in 293T cells expressing the let-7 sponge (Supplementary Fig. S2B), demonstrating let-7–mediated suppression of PD-L1. Accordingly, expression of the let-7 sponge increased PD-L1 protein on cell surface in MCF-7, U2OS, and Hela cells (Fig. 2A and B and Supplementary Fig. S2C). Overexpression of let-7 significantly decreased PD-L1 mRNA (Fig. 2C and D) and protein on cell surface (Fig. 2E and F and Supplementary Fig. S2D). These results demonstrate that let-7 negatively regulates expression of PD-L1 at the level of translation (i.e., posttranscriptionally). It is worth noting that different let-7 family members regulate PD-L1 in different cell lines, for example, let-7a, -7c, and -7e are most active in MCF-7 cells, whereas let-7d and let-7i are most active in U2OS cells.

LIN28 downregulates let-7 and promotes expression of PD-L1

LIN28 binds to let-7 pre-miRNA and blocks its maturation (15, 31). Consistent with this, knockdown of LIN28A or LIN28B by siRNA increased let-7 (Fig. 3A) and decreased PD-L1 on the cell surface of U2OS, MCF-7, and Hela cells (Fig. 3B and C and Supplementary Fig. S3A). However, overexpression of LIN28A/B significantly decreased let-7 (Fig. 3D) and increased cell-surface PD-L1 (Fig. 3E and F and Supplementary Fig. S3B). However, in LIN28A-overexpressed cells, overexpression of let-7c reduced PD-L1 to normal surface expression (Fig. 3G). Conversely, overexpression of LIN28A was able to rescue decreased PD-L1 by let-7c (Fig. 3H). Altogether, these results indicated that LIN28 regulates PD-L1 expression in a let-7–dependent manner.

Inhibition of LIN28 with C1632 decreases PD-L1 and inhibits cell growth

Malignant tumors frequently exhibit high expression of LIN28, and therefore low let-7 and high PD-L1 expression, suggesting that LIN28 and/or let-7 might be a therapeutic target to decrease PD-L1. Because different let-7 family members regulate PD-L1 in different cancer cells and all members are regulated by LIN28, LIN28 has a greater potential as a
Targetable candidate. A small-molecule inhibitor of LIN28, N-Methyl-N-[3-(3-methyl[1,2,4]triazolo[4,3-b]pyridazin-6-yl)phenyl]acetamide (C1632), was identified and reported to block LIN28’s activity to interact with let-7, thus rescuing the maturation of let-7 and increasing let-7 in cancer cells (32).

Our results showed that C1632 decreased LIN28 mRNA in U2OS, MCF-7, and HeLa cells (Fig. 4A–C) and increased let-7 in these human cancer cells (Fig. 4D–F) and mouse TUBO and 4T1 cancer cells (Supplementary Fig. S4A and S4B). As expected, cell-surface expression of PD-L1 decreased in human (Fig. 4G–I) and mouse cancer cells (Supplementary Fig. S4C and S4D) treated with C1632. Experiments repeated in multiple human cancer cell lines including MNNG/HOS, MG63, SAOS2, A549, and SKLU-1 confirmed the inhibition on PD-L1 expression by C1632 (Supplementary Fig. S5A–S5E). In addition, overexpression of LIN28 was able to rescue the inhibitory effect of C1632 on PD-L1 in U2OS cells (Supplementary Fig. S5F). These results support the idea that C1632 has the potential as an anticancer treatment that suppresses expression of PD-L1 by decreasing LIN28 expression and blocking interaction between LIN28 and let-7.

The effect of C1632 on cancer cell growth was also investigated. C1632 decreased the viability of U2OS cells at high dose (68 mg/L), but not at low dose (17 mg/L; Fig. 5A and B). Low dose of C1632 had a limited effect on cell growth, whereas at a high dose, C1632 inhibited cell proliferation without affecting cell morphology (Fig. 5C and D). Both flow cytometry and immunoblot assay revealed that C1632 treatment, even at a high dose, did not significantly increase cell apoptosis (Fig. 5E–G), suggesting limited cytotoxicity. The inhibitory effect of C1632 on cell viability and proliferation was also tested in MCF-7 (Supplementary Fig. S6A and S6B), Hela (Supplementary Fig. S6C and S6D), and A549 cells (Supplementary Fig. S6E and S6F). The results showed that although a low concentration of C1632 has no or limited effect on cell viability and proliferation, C1632 at high concentration significantly decreases cell viability and suppresses cell proliferation. Consistent with previous findings that low expression of PD-L1 decreases rate of cancer cell proliferation (33), depletion of PD-L1 by shRNA in TUBO cells (Supplementary Fig. S7A) showed moderate but consistent inhibition of cell growth (Supplementary Fig. S7B). It has also been reported that increased let-7 can suppress the proliferation of cancer cells in a PD-L1–independent manner (19). We thus proposed that C1632 treatment decreases PD-L1 by increasing let-7 and could inhibit cancer cell growth.

C1632 suppresses growth of syngeneic mammary fat pad tumors in mice

The above results prompted us to examine the effect of C1632 on syngeneic mammary fat pad tumors in immunocompetent mice.
mice, in order to determine whether C1632 could enhance endogenous antitumor activity in vivo. Recipient mice were injected with $5 \times 10^5$ TUBO cells prior to start of treatment. Five days later, tumor-bearing mice were randomly divided into two groups, and treated with C1632 or PBS (control) for 12 days. During treatment, no significant body weight loss was observed in control- or C1632-treated mice (Fig. 6A). Tumor size was measured every other day during treatment. The results showed that average tumor size was significantly smaller in immunocompetent mice treated with C1632 (Fig. 6B). As expected, tumors from C1632-treated mice had a lower expression of PD-L1 (Fig. 6C) than tumors from control mice. In addition, tumor-infiltrating CD8$^+$ T cells were more abundant (Fig. 6D; mean percentage of control group = 33.43 ± 2.36; C1632-treated group = 39.95 ± 4.82, $P = 0.026, n = 5$) and CD8$^+$ PD1$^+$ T cells were less abundant (Fig. 6E; mean percentage of control group = 29.77 ± 16.66, $P = 0.023, n = 5$) and tumor-infiltrating activated CD69$^+$ T cells were more abundant (Fig. 6F; mean percentage of control group = 9.10 ± 1.05; C1632-treated group = 12.54 ± 2.61, $P = 0.026, n = 5$) in C1632-treated mice than in control mice.

To further understand the inhibitory effect of C1632 on tumor growth, the above experiments were repeated using PD-L1-deficient TUBO cells (Supplementary Fig. S7A). We
observed that depletion of PD-L1 suppressed tumor growth with average tumor size decreasing from 1,432 to 856 mm³, and C1632 treatment further reduced average tumor size to 501 mm³ (Fig. 6B). These results suggested that in addition to its role on inhibition of PD-L1 in cancer cells, C1632 may have another function that enhances the tumor suppression.

It has been previously found that the blockade of PD-L1 in APCs is able to suppress tumor growth by enhancing T-cell activation (34). We thus speculated that C1632 may also inhibit PD-L1 in APCs, thereby promoting T cell–mediated antitumor immunity. Indeed, our experiments showed that expression of PD-L1 on cell surface (Supplementary Fig. S8D and S8E). Consistently, IFNγ and TNFα, indicators of activated immunity that are primarily secreted by T cells and APCs, respectively (35, 36), are significantly increased in response to C1632 treatment (Fig. 6G and H).

To further confirm that the immune system is required for C1632 induced tumor suppression, TUBO cells with or without PD-L1 were injected into immune-decient (BALB/c nu/nu) mice. We observed that there is no difference in tumor size regardless of PD-L1 or C1632 treatment (Fig. 6I), further demonstrating the requirement of T cell–mediated immunity in tumor suppression. Altogether, in vivo syngeneic tumor experiments suggested that C1632 inhibits tumor growth by stimulating T cell–mediated antitumor activity.
Figure 5.
Effects of C1632 on cancer cell growth. U2OS cells were grown for 12 days in the presence of medium containing C1632 (17 and 68 mg/L) or DMSO control. A, Schematic figure showing treatment with C1632 or DMSO control; cells were passaged every 4 days. B, After 4 days, cell viability was determined by cell viability assay. C, Population doublings were calculated during 12-day treatment with C1632 or DMSO control. D, Cell morphology was observed by light microscopy on the eighth day of treatment with C1632 or DMSO control. E, Cell apoptosis was quantified by flow cytometry. Representative images show apoptotic U2OS cells after 12-day treatment with the indicated dose of C1632. F, Quantification of E. Error bars, ± SD from mean value. P values were calculated using the Student t test (**, P < 0.01; ns, not significant). G, Immunoblot showing no increase of cleaved caspase-3 (marker of apoptosis) after 3-day treatment with C1632. Actin was used as an internal control.
Previous studies show that PD-1/PD-L1 inhibits antitumor immune surveillance and that agents that disrupt PD-1/PD-L1 stimulate this tumor defense mechanism. For example, humanized antibodies that interfere with PD-1/PD-L1 significantly improved clinical outcomes for human cancer patients, including patients with a wide range of malignancies. However, the response to antibody treatment was observed in fewer than 40% of patients, raising the possibility that antibody at the given dosage may not be sufficient to effectively block the interaction between PD-1 and PD-L1, especially when expression of PD-L1 is low (37, 38). Developing an alternative approach is thus desired.

Although many factors have been identified to regulate the expression of PD-L1 in cancer cells (39–41), few of them can be used as therapeutic targets due to lack of druggable molecules. In this report, we demonstrated that LIN28/let-7 regulates the post-transcriptional expression of PD-L1 and that C1632 is a small-molecule inhibitor of LIN28 that increases let-7, decreases PD-L1, and suppresses cell growth in a variety of human cancer cell types.

Discussion

Previous studies show that PD-1/PD-L1 inhibits antitumor immune surveillance and that agents that disrupt PD-1/PD-L1 stimulate this tumor defense mechanism. For example, humanized antibodies that interfere with PD-1/PD-L1 significantly improved clinical outcomes for human cancer patients, including patients with a wide range of malignancies. However, the response to antibody treatment was observed in fewer than 40% of patients, raising the possibility that antibody at the given dosage may not be sufficient to effectively block the interaction between PD-1 and PD-L1, especially when expression of PD-L1 is low (37, 38). Developing an alternative approach is thus desired. Although many factors have been identified to regulate the expression of PD-L1 in cancer cells (39–41), few of them can be used as therapeutic targets due to lack of druggable molecules. In this report, we demonstrated that LIN28/let-7 regulates the post-transcriptional expression of PD-L1 and that C1632 is a small-molecule inhibitor of LIN28 that increases let-7, decreases PD-L1, and suppresses cell growth in a variety of human cancer cell types. Results presented here also showed that C1632 suppresses

Figure 6.

Response of mammary fat pad tumor-bearing BALB/c mice to C1632 by tail-vein injection. Subcutaneous tumors were generated by injecting $5 \times 10^5$ TUBO cells with or without PD-L1 into mammary fat pad under #3 mammary gland of BALB/c mice. C1632 or PBS was delivered by intravenous tail-vein injection ($n=5$ for the PD-L1+ group and $n=3$ for the PD-L1− group). Body weight and tumor size were measured every 2 days. Twenty-four hours after the last injection, all mice were sacrificed. A, Body weight of mice treated with PBS or C1632. P value was calculated using an unpaired two-sided Student t test (ns, not significant). B, Tumors were excised on day 13 from mice treated with PBS or C1632. C, Flow cytometry MRI analysis showing relative cell-surface expression of PD-L1 in tumor cells. D–F, Tumor-infiltrating T cells were isolated, and the percentage of CD8+ T cells (D), CD8+PD1+ T cells (E), and CD69+ T cells (F) was determined by flow cytometry. G–H, Analysis of IFNγ (G) and TNFα (H) in blood serum from mice injected with PD-L1-depleted TUBO cells. I, TUBO cells with or without PD-L1 were injected into immune-deficient BALB/c mice. C1632 or PBS was delivered by intravenous tail-vein injection ($n=3$ per group). Tumor volume was measured every 2 days. Error bars, ±SD from the mean. P values were calculated by the Student t test (*, $P<0.05$; **, $P<0.01$).
progression of syngeneic tumors in vivo in immunocompetent mice and stimulates T-cell-mediated antitumor immune surveillance in these animals.

PD-L1 signaling induces T-cell exhaustion and alters frequency of tumor-infiltrating CD8⁺ T cells, whereas the blockade of PD-1/PD-L1 rescues T-cell functions and reactivates antitumor immune surveillance. Our results demonstrated that C1632 suppressed expression of PD-L1 in tumors, increased the percentage of tumor-infiltrating CD8⁺ T cells, decreased the frequency of CD8⁺PD-1⁺ T cells, and increased the frequency of infiltrating activated CD69⁺ T cells in tumor-bearing mice in vivo. Our results also showed that C1632 inhibited PD-L1 expression in THP-1 macrophages in vitro and increased TNFα and IFNγ in mice blood, suggesting that activated APCs may further enhance the antitumor immunity. Taken together, these results suggest that C1632 inhibits immune evasion and stimulates T-cell-mediated immune surveillance, and that it may be possible to exploit C1632 for therapeutic benefit toward human cancers. Because increased expression of LIN28 impairs T-cell development and leads to peripheral T-cell lymphoma (42), C1632 or other LIN28 inhibitors may be generally beneficial for T-cell maturation and function.

Previous studies demonstrate frequent upregulation of LIN28 in human tumors, and LIN28 has been proposed as a potential target for cancer therapeutics (42–44). LIN28 and let-7 influence a wide range of cellular processes including proliferation, metabolism, metastasis of cancer cells, and activation and maturation of APCs (45–48). Our results also demonstrated the inhibitory effect of C1632 on the viability and proliferation of cancer cells. Therefore, inhibition of LIN28 has dual functions for cancer therapy: suppressing tumor growth by inhibiting cancer cell proliferation and stimulating antitumor immune responses. Suppression of tumor growth may enhance the T-cell-to-tumor ratio and thus promotes antineoplastic immune activity, especially in tumors with reduced T-cell infiltration. Nevertheless, treatment with C1632 did not appear to significantly increase cell death (apoptosis). It remains to be determined whether C1632 or other inhibitors of LIN28 are well tolerated at therapeutically effective doses against cancer in humans.

C1632, which was first developed as an axiolytic agent (49), rescues maturation of let-7 by blocking the interaction between LIN28 and let-7 (32). Although our results showed that C1632 is able to decrease the expression of PD-L1 in vitro and in vivo, it is far from an ideal candidate for therapeutics. There is a need to develop a molecule that can inhibit LIN28 and rescue let-7 with much greater efficiency. Efforts to find derivatives of C1632 or other inhibitors that block the interaction between LIN28 and let-7 are warranted and would be facilitated by availability of the molecular structures of C1632 and LIN28/let-7 (50). Nonetheless, this study establishes that LIN28/let-7–mediated regulation of PD-L1 is a promising anticancer target that could be exploited in developing novel anticancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Chen, C. Xie, Y. Zhao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Chen, X. Zheng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Chen, C. Xie, X. Zheng, Y. Zhao
Writing, review, and/or revision of the manuscript: Y. Chen, C. Xie, Y. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Chen
Study supervision: Y. Chen, Y. Zhao

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Targeting LIN28/let-7 for PD-L1-Mediated Immunotherapy

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