Improving PD-1 blockade plus chemotherapy for complete remission of lung cancer by nanoPDLIM2

Fan Sun[1], Pengrong Yan[1], Yadong Xiao[1, 2, 3, 4], Hongqiao Zhang[3], Steven D. Shapiro[2, 4], Gutian Xiao[1, 3, *], and Zhaoxia Qu[1, 3, *]

[1]UPMC Hillman Cancer Center, Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA. [2]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA. [3]Norris Comprehensive Cancer Center, Hastings Center for Pulmonary Research, Department of Molecular Microbiology and Immunology, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA. [4]Department of Medicine, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA.

[*]Correspondence should be addressed to Zhaoxia Qu (Email: Zhaoxia.Qu@med.usc.edu; Phone: 323-442-6932) or Gutian Xiao (Email: Gutian.Xiao@med.usc.edu; Phone: 323-442-7985)

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Abstract

Background: Immune checkpoint inhibitors (ICIs) and their combination with other therapies such as chemotherapy, fail in most cancer patients. We previously identified the PDZ-LIM domain-containing protein 2 (PDLIM2) as a *bona fide* tumor suppressor that is repressed in lung cancer to drive cancer and its chemo and immunotherapy resistance, suggesting a new target for lung cancer therapy improvement.

Methods: Human clinical samples and data were used to investigate *PDLIM2* genetic and epigenetic changes in lung cancer. Using an endogenous mouse lung cancer model faithfully recapitulating refractory human lung cancer and a clinically feasible nano-delivery system, we investigated the therapeutic efficacy, action mechanism, and safety of systemically administrated PDLIM2 expression plasmids encapsulated in nanoparticles (nanoPDLIM2) and its combination with PD-1 antibody and chemotherapeutic drugs.

Results: PDLIM2 repression in human lung cancer involves both genetic deletion and epigenetic alteration. NanoPDLIM2 showed low toxicity, high tumor specificity, antitumor activity, and greatly improved the efficacy of anti-PD-1 and chemotherapeutic drugs, with complete tumor remission in most mice and substantial tumor reduction in the remaining mice by their triple combination. Mechanistically, nanoPDLIM2 increased major histocompatibility complex class I (MHC-I) expression, suppressed multi-drug resistance 1 (MDR1) induction and survival genes and other tumor-related genes expression in tumor cells, and enhanced lymphocyte tumor infiltration, turning the cold tumors hot and sensitive to ICIs and rendering them vulnerable to chemotherapeutic drugs and activated tumor-infiltrating lymphocytes (TILs) including those unleashed by ICIs.

Conclusions: These studies established a clinically applicable PDLIM2-based combination therapy with great efficacy for lung cancer and possibly other cold cancers.

Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women with a 5-year survival rate of only 22% [1]. Although these outcomes may be improved by immune checkpoint blockade therapy involving the disruption of the binding of programmed cell death 1 (PD-1, also known as CD279) on tumor-infiltrating lymphocytes (TILs) to programmed death receptor ligand 1 (PD-L1, also known as B7-H1 or CD274) on tumor and tumor-associated cells, most lung cancer patients still fail the therapy, with a response rate of only about 20% [2]. In general, this revolutionary immunotherapy works better against 'hot' tumors, which have abundant TILs, strong immunogenicity and sufficient PD-L1 expression. Unfortunately, most tumors are 'cold', with low T-cell infiltration, weak immunogenicity and minimal PD-L1 expression, and show weak response to immune checkpoint inhibitors (ICIs) [3, 4].

Cold lung tumors without targetable oncogenic drivers are treated with chemotherapy as the standard approach [5]. However, the response rate to this conventional cancer therapy is also low and resistance often occurs after an initial response, with an overall survival (OS) of about 12-17 months. Given its roles in inducing TILs and immunogenicity, in particular PD-L1 expression, to turn cold tumors hot, chemotherapy can be an ideal candidate for combination with PD-1/PD-L1 blockade to improve therapeutic efficacy [6]. Indeed, combination treatment with ICIs and chemotherapeutic drugs shows synergy and better efficacy in both preclinical animal models and clinical trials of lung cancer and other cancers [6-8]. However, even with the combination of ICIs and chemotherapy, tumors in animals do not remit completely and the objective response rate (ORR) of lung cancer patients only reaches 33%-49.7%, with a median progression-free survival (PFS) of just 5.1-9 months and a median OS of 13-22 months [7, 8]. Thus, further improvement over the chemo-immunotherapy is direly needed.

Our recent human and mouse studies have shown that most lung tumors not only have low TILs and

decreased PD-L1, but also down-regulate major histocompatibility complex class I (MHC-I), evading recognition and attack by $CD8^+$ T cells, including those unleashed by ICIs and/or recruited by chemotherapy [6, 9, 10]. Following our previous cell line studies [11-17], we have established the PDZ-LIM domain-containing protein PDLIM2, also known as SLIM or mystique [18-20], as a *bona fide* tumor suppressor and its repression as a causative driver of lung cancer and resistance to ICIs and chemotherapeutic agents [6]. While PDLIM2 is epigenetically repressed in human lung cancer, associating with therapeutic resistance and poor prognosis, its global or lung epithelial-specific deletion in mice leads to lung cancer development, chemoresistance, and complete resistance to anti-PD-1 and epigenetic drugs. One most important function of PDLIM2 is to promote the ubiquitination and proteasomal degradation of nuclear signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) RelA (also known as p65), two master transcription factors that function as proto-oncogenes in lung and many other cancers [6, 10-13, 21-27]. PDLIM2 repression in tumor cells thus results in the persistent activation of STAT3 and RelA, leading to MHC-I downregulation and high expression of tumor growth-related genes [6]. It also leads to strong induction of multi-drug resistance 1 (MDR1) for acquired chemo-resistance, as chemotherapy further enhances RelA activation for MDR1 transcription in PDLIM2 deficient tumor cells [6].

In this study, we examined whether and how PDLIM2 can be targeted to treat lung cancer in a faithful mouse model of human lung cancer. In particular, we tested whether systemic administration of nanoparticle-encapsulated PDLIM2-expression plasmids (nanoPDLIM2) could enhance the efficacy of anti-PD-1 and/or chemotherapeutic drugs. We also examined whether PDLIM2 repression in human lung cancer involves genetic deletion and its relationship with epigenetic silencing. These studies indicate that besides epigenetic repression, loss of heterozygosity (LOH) contributes to *PDLIM2* downregulation in about 58% of human lung tumors, and that *Pdlim2* heterozygous deletion (*Pdlim2*$^{+/-}$) mice develop spontaneous tumors in lung and other organs. Notably, systemic administration of

nanoPDLIM2 reverses the phenotypes caused by PDLIM2 repression and induces complete remission of all lung tumors in most mice without further increasing toxicity when combined with anti-PD-1 and chemotherapeutic drugs. These findings provide a firm basis to combine ICIs and chemotherapeutic drugs with PDLIM2-targeted therapy for the treatment of lung and other cancers.

Material and Methods

Animals and lung carcinogenesis

We have complied with all relevant ethical regulations for animal testing and research. The animal experiments were performed in accordance with the US National Institutes of Health (NIH) Guidelines on the Use of Laboratory Animals. All animals were maintained under pathogen-free conditions and used according to protocols approved by the Institutional Animal Care and Use Committee (IACAUC) of the University of Pittsburgh. *Pdlim2*$^{flx/flx}$/*SP-C*-rtTA$^{tg/-}$/(tetO)7CMV-Cre$^{tg/tg}$ (ΔSPC) mice under a pure FVB/N background and *Pdlim2*$^{-/-}$ mice under a pure BALB/c background have been described before [6, 19, 21, 22, 28]. For lung carcinogenesis, six- to eight-week-old ΔSPC and wild type FVB/N mice were intraperitoneally (i.p.) injected with urethane (1 mg/g body weight, Sigma-Aldrich, St. Louis, MO, USA) once a week for six consecutive weeks [6], followed by different treatments as shown in the figures. Mice were euthanized at six weeks post urethane treatment for examination of lung tumors, inflammation, and treatment-induced toxicity. Surface tumors in mouse lungs were counted blinded under a dissecting microscope, and tumor diameters were measured by microcalipers.

Preparation of PDLIM2-expression plasmid or empty vector plasmid nanoparticles

The polyethylenimine (PEI)-based nanoparticles (*in vivo*-jetPEI) (Polyplus Transfection, New York, NY, USA) and plasmid DNA complexes at a nitrogen-to-phosphate ratio of 8 (N/P=8) were prepared according to the manufacturer's instructions. Briefly, 25 μg of pCMV-myc-*Pdlim2* or empty vector

plasmids in 100 μl of a 5% glucose solution were mixed with the *in vivo*-jetPEI reagent (4 μl) diluted into 100 μl of a 5% glucose solution. After 15 minutes of incubation at room temperature, the mixed solution (200 μl/mouse) was injected intravenously (i.v.) via the tail vein. PDLIM2-expression plasmid or empty vector plasmid DNA in tissues were measured by PCR assays targeting Amp-R in the genome of these plasmids.

Histology and immunohistochemistry (IHC) analysis

Lung, liver, kidney, and spleen tissues were excised, fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections. Sections were stained with H&E or subjected to sequential incubations with the indicated primary antibodies, biotinylated secondary antibodies and streptavidin-HRP [6]. Antibodies were listed in Supplementary Table S1.

***In vivo* BrdU labeling**

Mice were i.p. injected with 50 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO, USA) 24 h prior to euthanasia. Mouse lung tissue sections were stained with anti-BrdU (Sigma-Aldrich, St. Louis, MO, USA). BrdU labeling index was calculated as the percentage of labeled cells per total cells counted (> 500 cells in each counted tumor-containing area).

Flow cytometry (FACS) analysis

The cells were isolated from mouse lungs using collagenase/dispase digestion followed by filtration with 70 μm Nylon cell strainer and red blood cell lysis; then the cells were incubated with the antibodies against cell surface antigens after blocked with αCD16/CD32. The cells were then fixed with paraformaldehyde (2%), permeabilized and incubated with antibodies against intracellular antigens if needed. For interferon-γ (IFNγ) staining, cells were treated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (1 μM), brefeldin A (BFA, 3 μg/ml) and monensin (2 μM) for 4 h before

they were stained for FACS analysis. Data were acquired and analyzed by Accuri C6 or LSRFortessa I (BD Biosciences) and FlowJo software [29].

Quantitative polymerase chain reaction (qPCR) analysis

The indicated tissues or cells were subjected to DNA or RNA extraction, RNA reverse transcription and real-time PCR using trizol, reverse transcriptase, and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA) according to manufacturer's protocol. Primer pairs used for qPCR were listed in Supplementary Table S2.

Microsatellite and gene-specific PCR-based LOH analysis of *PDLIM2*

Genomic DNAs were isolated from human lung tumors and their matched normal tissues using the PureLink Genomic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA), and subjected to semi-quantitative PCR using the primers specific for the microsatellite markers D8S1786 and D8S1752 that straddle the *PDLIM2* genetic locus or the *PDLIM2* genetic locus itself. Primer pairs used for the assays were listed in Supplementary Table S2.

Statistical analysis

One-way ANOVA power analysis was used to determine the minimum sample size. Animals were randomly assigned to different treatment groups. Measurements were taken from distinct samples. Student's t test (two tailed) and one-way ANOVA/Tukey's or two-way ANOVA/Sidak's test were used to assess significance of differences between two groups and multiple comparisons, respectively. Pearson's correlation test was used to assess association between *PDLIM2* expression with its promoter methylation or genetic deletion and the overlap between *PDLIM2* promoter methylation and genetic deletion. All bars in figures represent means ± standard error of the mean (SEM). The *p* values are indicated as $*p < 0.05$, $**p < 0.01$, ns, not statistically significant, except for those shown in figures.

The p values < 0.05 and 0.01 are considered statistically significant and highly statistically significant, respectively. Grubbs' and ROUT outlier tests were established a priori. There were no exclusions in the analyses.

Results

Both epigenetic repression and genetic deletion of *PDLIM2* in lung cancer

Using 40% of the expression level in matched normal lung tissues as the cut-off in the analysis of the Cancer Genome Atlas (TCGA) data, we found that *PDLIM2* was repressed in over 75% of human lung tumors in a prior study [6]. If using 50% as the cut-off, *PDLIM2* was repressed in about 93% of human lung tumors (Figure 1*A*). Using 125% of the methylation level of the *PDLIM2* promoter in normal lung tissues as the cut-off to analyze TCGA database, over 70% of human lung tumors were found to have hypermethylation of the *PDLIM2* promoter (Figure 1*A*, 1*B*), further validating our previous finding of epigenetic silencing as the main mechanism underlying PDLIM2 repression [6].

In line with *PDLIM2*'s location on chromosome 8p21.3, a frequent LOH region in lung and other tumors [30-34], analysis of TCGA database revealed that *PDLIM2* expression was positively associated with its gene copy numbers, and that over 58% of human lung tumors had genetic deletion of the *PDLIM2* gene if copy number variation (CNV) of -0.1 was used as the cut-off for the gene deletion (Figure 1*C*). Further analysis indicated that about 44% of human lung tumors simultaneously harbored the promoter hypermethylation and LOH of the *PDLIM2* gene, and approximately 26% and 14% of them only having the promoter hypermethylation or LOH of the *PDLIM2* gene, respectively (Figure 1*D*). Around 16% of lung tumors possessed no such epigenetic or genetic alterations in *PDLIM2*. These findings were confirmed by microsatellite and gene-specific PCR-based LOH analysis of human primary lung tumor tissues and cell lines (Figure 1*E*, 1*F*; Supplementary Table S3). These data suggested that PDLIM2 repression in lung cancer involves both epigenetic silencing and genetic deletion.

To determine the significance of *PDLIM2* LOH in lung tumors, we examined whether *Pdlim2* heterozygous deletion leads to decreased PDLIM2 expression and spontaneous tumors in mice. Unlike its absolute absence in *Pdlim2* homozygous deletion (*Pdlim2*$^{-/-}$) mice, PDLIM2 was detected in the lung of *Pdlim2*$^{+/-}$ mice, but at a much lower level compared to wild type (WT) mice (Supplementary Figure S1*A*). Importantly, *Pdlim2*$^{+/-}$ mice, like *Pdlim2*$^{-/-}$ mice, also developed spontaneous tumors (Figure 1*G*). Of note, over 50% of tumors developed in *Pdlim2*$^{-/-}$ and *Pdlim2*$^{+/-}$ mice were lung tumors (Figure 1*H*; Supplementary Figure S1*B*). These data are also highly consistent with the fact that PDLIM2 is ubiquitously expressed under physiological conditions, with the highest level in the lung and lung epithelial cells in particular [6, 18-20]. Thus, PDLIM2 is a haploinsufficient tumor suppressor that is particularly important for lung tumor suppression**.**

Efficacy of systemic administration of nanoPDLIM2 in refractory lung cancer

Although reversal of PDLIM2 epigenetic repression by epigenetic drugs to restore PDLIM2 expression in cancer cells may be used to treat lung cancer [6], it is logical that this approach cannot be applied to lung tumors involving *PDLIM2* LOH, which accounts for about 58% of all lung cancer cases. To overcome this limitation and expand PDLIM2-targeted therapy to all lung tumors with PDLIM2 repression regardless of the involved mechanisms, we tested the therapeutic efficacy of systemic administration of PDLIM2-expression plasmids encapsulated by the clinically feasible *in vivo*-jetPEI [35-39]. To this end, we employed mouse lung tumors induced by urethane, a faithful model of human lung cancer and adenocarcinoma (AC) in particular [6, 23, 24, 40-43]. Urethane is a chemical carcinogen present in fermented food, alcoholic beverage and also cigarette smoke, the predominant risk factor accounting for about 90% of human lung cancer cases [44]. Like its human counterpart, the murine lung cancer induced by urethane also shares PDLIM2 repression, in addition to their similarities in histology, genetics, molecular biology and immunology [6, 9, 23, 24, 40-43]. WT mice with lung

tumors induced by urethane were i.v. injected with nanoparticle-encapsulated *Pdlim2* plasmids or empty vector plasmids. Six weeks post the initial treatment of nanotherapy, mice were euthanized and lung tissues were collected (Figure 2*A*). Compared to the vector plasmid mock-treated group (Vec), nanoPDLIM2-treated mice had a significantly reduced tumor burden in their lungs, although the decrease in tumor numbers was not statistically significant (Figure 2*B*).

In line with the tumor reduction, nanoPDLIM2 administration decreased nuclear RelA and STAT3, a hallmark of NF-κB and STAT3 activation, accordingly reduced the expression of their downstream cell survival gene Bcl-xL and cell proliferation gene Cyclin D1, increased apoptosis and decreased proliferation of lung cancer cells (Figure 2*C*-2*E*). These data indicated that intravenous administration of nanoPDLIM2 shows efficacy in suppressing oncogenic RelA and STAT3 activation and in treating lung cancer.

High tumor specificity and low toxicity of systemic administration of nanoPDLIM2

To characterize the potential new lung cancer therapy, we examined the expression levels of PDLIM2 in lung tumors and several organs, including the liver, kidney, and spleen. Consistent with the treatment efficacy, a high level of PDLIM2 was detected in the lung tumors from mice treated with the PDLIM2 plasmid nanoparticles one week post nanoPDLIM2 treatment, whereas no obvious PDLIM2 was found in the lung tumors from mice treated with the control plasmid nanoparticles (Figure 3*A*, 3*B*). It should be pointed out that the tumor delivery efficiency of PDLIM2-expression or control plasmid nanoparticles was similarly high (Figure 3*B*). However, either PDLIM2-expression or control plasmids were hardly detected in other organs/tissues of the same mice, including liver, kidney and spleen. Of note, in the first two days after injection, both empty vector and PDLIM2-expression plasmids were also detected at high levels in those tissues but quickly cleared afterward (data not shown), consistent with the well-documented tumor-specific enrichment of nanoparticles [37, 45-47]. Consistently, ectopic

Pdlim2 was not detected, and the levels of PDLIM2 proteins were comparable in these tissues (Figure 3*B*, 3*C*).

In further support of the lung tumor-specific delivery, the i.v. injection of either PDLIM2-expression or control plasmid nanoparticles showed no obvious toxicity to animals, as evidenced by no significant changes in the animal body weight and histology of all organs/tissues we examined, including spleen, kidney and liver (Figure 3*D*, *E*). Mouse appearance and behaviors, such as eating, drinking, defecating, urinating, sniffing, grooming, and digging, were not different between PDLIM2-expression and control plasmid nanoparticle groups (data not shown). Taken together, these data suggested the therapeutic efficacy and low toxicity of PDLIM2-based nanotherapy in the mouse model of refractory lung cancer.

Synergy of nanoPDLIM2 with chemotherapy in lung cancer treatment

Given the role of PDLIM2 in inhibiting the expression of cell survival and proliferation genes in tumor cells, which contribute to chemoresistance, we tested whether nanoPDLIM2 increases the efficacy of chemotherapy in the mouse model of lung cancer. Treatment with carboplatin and paclitaxel, two chemotherapeutic drugs that are often used together as the first-line treatment for lung and many other cancers, led to significant decrease in tumor number and tumor burden (Figure 4*A*). Importantly, combination with nanoPDLIM2 further significantly decreased both tumor number and tumor burden, suggesting a promising synergy between PDLIM2 nanotherapy and chemotherapy in lung cancer treatment. Consistently, significantly higher tumor cell apoptosis was detected in mice treated with the combination therapy, in comparison to those with nanoPDLIM2 or chemotherapy alone (Figure 4*B*).

Another important mechanism contributing to the synergy between these two therapies involves nanoPDLIM2 blockade of the acquired chemoresistance of lung cancer cells. Chemotherapy induced strong RelA activation/nuclear expression and MDR1 expression (Figure 4*C*, 4*D*). NanoPDLIM2 not

only repressed the constitutive activation of RelA in cancer cells but also prevented the strong induction of RelA activation and MDR1 expression by the chemotherapy. Thus, PDLIM2 nanotherapy improves the therapeutic efficacy of chemotherapy through blocking both intrinsic and acquired chemoresistance of lung cancer cells.

NanoPDLIM2 enhancement of ICI's efficacy in lung cancer treatment

High expression of cell survival genes also renders tumor cells resistant to the tumoricidal activity of cytotoxic T lymphocytes (CTLs), including those unleashed by ICIs. PDLIM2 nanotherapy should also enhance the efficacy of immunotherapy, given its ability in suppressing the expression of cell survival genes. Furthermore, NanoPDLIM2 increased the number of TILs and the expression of MHC-I, the most important and core components of immunotherapies including PD-1 immune checkpoint blockade therapy (Figure 5*A*, 5*B*). In line with our previous studies [6], PD-1 blocking antibody showed some efficacies in the mouse model of lung cancer, as evidenced by the significant decrease in tumor burden and tumor number (Figure 5*C*). Combining with PDLIM2 nanotherapy further significantly decreased tumor burden, although the decrease in tumor number failed to reach statistical significance. Consistently, significant increases in both $CD4^+$ and $CD8^+$ TILs, $CD8^+$ CTL activation and lung tumor cell death were detected (Figure 5*D*-5*F*).

Complete remission of all lung tumors in most mice by the combination treatment of nanoPDLIM2 and anti-PD-1 and chemotherapeutic drugs

Like most human lung tumors, lung tumors in our animal model exhibit decreased expression of PD-L1 on cell surface, in additional to the low expression of MHC-I and low number of TILs [6, 9]. In line with our previous finding that PD-L1 expression is largely independent of PDLIM2 [6], nanoPDLIM2 failed to induce PD-L1 expression in lung tumors, which was in contrast to chemotherapy (Figure 6*A*).

This may explain why the enhancing effect of nanoPDLIM2 on PD-1 blockade therapy is only moderate.

Although it dramatically increases TILs as well [6], chemotherapy also induced the expression of PD-L1 on cancer cells (Figure 6*A*), which presumably protects cancer cells from immune attack and thereby restricts further efficacy improvement of its combination with PDLIM2 nanotherapy. But it may increase the sensitivity of cancer cells to PD-1 blockade therapy, particularly in combination with nanoPDLIM2. Indeed, chemotherapy and PD-1 blockade therapy showed a promising synergy in reducing both tumor number and tumor burden in comparison to the individual treatment of chemotherapy or PD-1 blockade therapy [6]. The synergy was largely blocked when *Pdlim2* was genetically deleted from lung cancer cells (ΔSPC) (Figure 6*B*, 6*C*), suggesting an important role of PDLIM2 in the combination therapy of chemotherapeutic drugs and anti-PD-1. Whereas PDLIM2 nanotherapy increased MHC-I expression on tumor cells (Figure 5*B*), which is critical for better recognition and killing of tumor cells by $CD8^+$ CTLs, chemotherapy alone or its combination with anti-PD-1 failed to do so (Figure 6*D*). Accordingly, combination of anti-PD-1 and chemotherapeutic drugs, like the combination therapies of nanoPDLIM2 and anti-PD-1 or chemotherapeutic drugs, also failed to induce a complete remission of lung tumors in any mice.

Given their overlapping roles in increasing TILs and in particular their complement roles in inducing MHC-I and PD-L1 expression on tumor cells, which turn tumors hot and more sensitive to PD-1 blockade, combination of nanoPDLIM2 and chemotherapeutic drugs with anti-PD-1 is expected to show better efficacy compared to combining only two of them. Indeed, combination of all three showed the strongest effect on reducing tumor burden and numbers, and caused complete remission of all lung tumors in 60% of mice (Figure 6*E*) and much more shrinkage of lung tumors in remaining mice in comparison to those treated with two combined therapies. In line with the high therapeutic efficacy, the

triple combination therapy significantly increased the numbers and/or activation of $CD4^+$ and $CD8^+$ T cells in the lung, compared to the combination of chemotherapeutic drugs and anti-PD-1 (Figure 6*F*, 6*G*).

Of note, consistent with the undetectable toxicity of PDLIM2 nanotherapy, its co-treatment did not further increase the toxicity of anti-PD-1 and chemotherapeutic drugs. There was no obvious histological difference of major organs, including the liver, lung, kidney, and spleen (Supplementary Figure S2). Moreover, no significant differences in animal body weights were observed by additional nanoPDLIM2, in comparison to the mice received the combinational treatment of anti-PD-1 and chemotherapeutic drugs in the presence or absence of empty vector plasmid nanoparticles (Figure 6*H*). These data suggested a novel combination therapy with very high therapeutic efficacy and no increased toxicity for lung cancer, particularly refractory lung cancer.

Discussion

PD-1/PD-L1 blockade immunotherapy has recently joined chemotherapy as a standard treatment for lung and several other cancers [2-4]. While some patients have shown dramatic responses, most patients do not benefit from this novel treatment. Currently, various combinations of ICIs with other therapies, in particular its combination with chemotherapeutic drugs are being extensively tested in both preclinical and clinical trial studies to expand the benefit of this innovative therapy [6-8]. Although a promising synergy and better efficacy has been observed in both preclinical animal models and human clinical trial studies [6-8], significant further improvement is direly needed. Using an authentic mouse model of lung cancer, we show, for the first time, that PDLIM2 nanotherapy shows efficacy and high safety, and more importantly, induces complete remission of all lung tumors in most animals when it is combined with anti-PD-1 and chemotherapeutic drugs.

Most human lung tumors as well as lung tumors in our animal model have low numbers of TILs and decreased expression of PD-L1 and MHC-I on the cell surface [6, 9], all of which are important mechanisms contributing to the resistance to PD-1 blockade therapy. Through inducing immunogenic cell death (ICD) of cancer cells [8], chemotherapy can increase TILs and PD-L1 expression on tumor cells, and thereby synergize with anti-PD-1. However, chemotherapy cannot induce MHC-I expression, which limits further improvement of its synergy with immune therapy for complete cancer remission. Also, tumor cells usually express high levels of survival genes against the tumoricidal effects of chemotherapeutic drugs and of CTLs, including those activated by chemotherapy and unleashed by ICIs.

On the other hand, PDLIM2 nanotherapy induces MHC-I expression and lymphocyte tumor infiltration but does not up-regulate PD-L1. Moreover, PDLIM2 nanotherapy prevents the induction of MDR1 and the expression of tumor-related genes and in particular cell survival genes, further sensitizing tumor cells to the cytotoxicity of chemotherapeutic drugs and immune cells including those recruited by chemotherapy and unleashed by PD-1 blockade. Because of these important functions of PDLIM2 nanotherapy, PDLIM2 nanotherapy improved the efficacy of chemotherapy and PD-1 blockade therapy, and in combination with chemotherapy and PD-1 blockade therapy, resulted in complete cancer remission in most of the animals and dramatic tumor reduction in the remaining mice.

Another important clinical characteristic of PDLIM2 nanotherapy is its tumor-specificity and high safety profile. It delivered PDLIM2-expression plasmids to lung tumor tissues and showed undetectable toxicity in the animal model. Its combination does not further increase the toxicity of anti-PD-1 and chemotherapeutic drugs. This is in sharp contrast to the Food and Drug Administration (FDA)-approved epigenetic drugs, which can restore PDLIM2 expression in cancer cells with PDLIM2 epigenetic repression [6, 10-15]. Epigenetic drug treatment leads to body weight loss of animals (Supplementary

Figure S3*A*). The toxicity is much worse when epigenetic drugs are combined with chemotherapy (Supplementary Figure S3).

Besides its side effects, epigenetic therapy should have much more limited application, even though it shows better efficacy as a monotherapy or combined with chemotherapeutic drugs or anti-PD-1 in comparison to PDLIM2 nanotherapy (Supplementary Figure S4). While nanoPDLIM2-based combination therapies could be applicable to all lung tumors with PDLIM2 repression regardless of the mechanisms involved, epigenetic therapy may be used to treat about 26% of lung tumors with PDLIM2 epigenetic repression only. About 58% of lung tumors harboring PDLIM2 LOH are not suitable to epigenetic therapies, although most of them are also with epigenetic alterations of the *pdlim2* gene. Of note, the therapeutic efficacy of epigenetic therapy depends on PDLIM2 expression [6], and PDLIM2 heterozygous loss causes spontaneous lung and other cancers. Further, the efficacy and toxicity of the combination of epigenetic agents with both anti-PD-1 and chemotherapeutic drugs has not been examined yet.

Conclusions

In summary, the presented data identify genetic deletions as a major mechanism other than epigenetic alterations for PDLIM2 repression in human lung cancer, and PDLIM2 as a haploinsufficient tumor suppressor particularly important for suppressing lung cancer and therapy resistance. More importantly, these preclinical data establish a novel combination treatment of nanoPDLIM2, anti-PD-1 and chemotherapeutic drugs that induces complete remission of all lung tumors in most animals and is also with high safety profile. We believe that these knowledges are applicable to other cancers, because PDLIM2 repression has also been linked to numerous human cancers other than lung cancer.

Abbreviations

AC: adenocarcinoma; BFA: brefeldin A; CNV: copy number variation; CTL: cytotoxic T lymphocyte; FACS: fluorescent activated cell sorting; FDA: Food and Drug Administration; IFNγ: interferon-γ; ICD: immunogenic cell death; ICI: immune checkpoint inhibitor; IHC: immunohistochemistry; i.p.: intraperitoneal; i.v.: intravenous; LOH: loss of heterozygosity; MDR1: multi-drug resistance 1; MHC-I: major histocompatibility complex class I; nanoPDLIM2: nanoparticle-encapsulated PDLIM2-expression plasmids; NF-κB: nuclear factor-κB; NIH: National Institutes of Health; ORR: objective response rate; OS: overall survival; PD-1: programmed cell death 1; PD-L1: programmed death ligand 1; PD-L2: programmed death ligand 2; PDLIM2: PDZ-LIM domain-containing protein 2; PEI: polyethylenimine; PFS: progression-free survival; PMA: phorbol 12-myristate 13-acetate; qPCR: quantitative polymerase chain reaction; SEM: standard error of the mean; STAT3: signal transducer and activator of transcription 3; TCGA: The Cancer Genome Atlas; TIL: tumor-infiltrating lymphocyte; WT: wild type.

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Availability of data and materials

TCGA lung adenocarcinoma, lung squamous cell carcinoma, and lung cancer data we analyzed were obtained from https://portal.gdc.cancer.gov/projects. All data generated or analyzed during this study are included in this article and its additional files. Extra data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author contributions

Z.Q. and G.X. conceived and designed the study, led and contributed to all aspects of the analysis, and wrote the manuscript. S.D.S. contributed to the overall experimental design and data analysis and interpretation, provided advice and constructive feedback, and edited the manuscript. F.S. performed and analyzed all the experimental assays. H.Z. contributed to data analysis and edited the manuscript. P.Y. originally identified PDLIM2 LOH in human lung tumors by gene-specific PCR-based LOH analysis. Y.X. contributed to mouse clone maintenance and IHC staining.

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and carried out in accordance with NIH guidelines on animal care.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. PDLIM2 repression in human lung cancer involves both epigenetic alteration and genetic deletion, and *Pdlim2* genetic deletion in mice leads to development of spontaneous tumors, majorly lung tumors. (A) TCGA data showing *PDLIM2* repression in over 90% of lung tumors if using 50% of the expression level in normal lung tissues as the cut-off (NL, n = 110; Tumor, n = 1019). (B) TCGA data showing *PDLIM2* promoter hypermethylation and expression repression (dashed box) in over 70% of lung tumors when using 125% of the methylation level in normal lung tissues as the cut-off (n = 827). (C) TCGA data showing positive associations between *PDLIM2* expression and its gene copy numbers as well as *PDLIM2* genetic deletion and expression repression (dashed box) in about 58% of lung tumors using the copy number variation of -0.1 as the cut-off (n = 1010). (D) TCGA data showing simultaneous promoter hypermethylation and genomic deletion of *PDLIM2* (dashed box) in about 44% of lung tumors (n = 816). (E) Microsatellite-PCR showing *PDLIM2* loss in human lung tumors (n = 21). (F) qPCR showing *PDLIM2* loss in human lung cancer cell lines with known copy number of the *PDLIM2* gene (n = 25). (G) Kaplan-Meier tumor-free survival curve showing increased spontaneous tumors in *Pdlim2*$^{-/-}$ and *Pdlim2*$^{+/-}$ mice compared to WT mice. Gehan-Breslow-Wilcoxon test was performed. $*p < 0.05$. (H) Percentage of tumor types spontaneously developed in *Pdlim2*$^{-/-}$ and *Pdlim2*$^{+/-}$ mice showing a majority of lung tumors.

Figure 2. Systemic administration of PDLIM2 plasmid nanoparticles shows efficacy in mouse model of refractory lung cancer. (A) Schedule of lung cancer induction and treatment. (B) Urethane model showing efficacy of intravenous administration of PDLIM2-expression plasmid nanoparticles for refractory lung cancer ($n \geq 6$). Nanoparticles with an empty vector plasmid (Vec) that was employed to express PDLIM2 were used as a control. (C) IHC staining showing decreased nuclear expression of STAT3 and RelA in lung tumors by PDLIM2 nanotherapy (n = 6). (D) IHC staining showing decreased Bcl-xL and increased apoptosis marker cleaved caspase -3 in lung tumors by PDLIM2 nanotherapy (n =

6). (E) IHC staining showing decreased Cyclin D1 and proliferation (BrdU incorporation) in lung tumors by PDLIM2 nanotherapy (n = 6). Scale bar in (C-E), 20 μm. Student's *t* test was performed (two tailed, unpaired) and data represent means ±SEM in (B-E). $^{**}p < 0.01$; ns, not statistically significant.

Figure 3. PDLIM2 nanotherapy shows high tumor specificity and low toxicity. (A) IHC staining showing high PDLIM2 re-expression in lung tumors after PDLIM2 nanotherapy. (B) PCR and IB assays showing lung tumor-specific plasmids delivery and PDLIM2 expression by PDLIM2 nanotherapy (n = 3). (C) IHC staining showing comparable expression of PDLIM2 in the indicated tissues of mice treated with PDLIM2 expression plasmid or empty vector plasmid nanoparticles. (D) No significant changes in animal body weight by nanoPDLIM2 (n = 5). (E) H&E staining showing no noticeable changes in major organs by nanoPDLIM2. Scale bar: (A and C) 20 μm; (E) 50 μm.

Figure 4. PDLIM2 nanotherapy renders lung cancers more vulnerable to chemotherapy. (A) Urethane model showing synergy of PDLIM2 nanotherapy and chemotherapy in lung cancer treatment ($n \geq 5$). (B) IHC staining showing increased lung tumor cell apoptosis by PDLIM2 nanotherapy, chemotherapy, and further increase by their combination (n = 6). (C) IHC staining showing RelA activation by chemotherapy and blockage of chemo activation of RelA by PDLIM2 nanotherapy (n = 6). (D) IHC staining showing strong MDR1 induction by chemotherapy and blockage of MDR1 induction by PDLIM2 nanotherapy (n = 6). Scale bar in (B-D), 20 μm. Student's *t* test was performed (two tailed, unpaired) and data represent means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not statistically significant.

Figure 5. PDLIM2 nanotherapy increases the efficacy of PD-1 blockade immunotherapy for refractory lung cancer. (A) IHC staining showing increased TILs in lung tumors by PDLIM2 nanotherapy (n = 6).

(B) FACS showing increased MHC-I expression in lung tumor cells by PDLIM2 nanotherapy (n = 4). (C) Urethane model showing PDLIM2 nanotherapy enhancing PD-1 immunotherapy efficacy in lung cancer treatment (n ≥ 5). (D) IHC staining showing increased TILs by PDLIM2 nanotherapy in the context of immunotherapy (n = 6). (E) FACS showing increased activation of $CD8^+$ T cells by PDLIM2 nanotherapy in the context of immunotherapy (n = 3). (F) IHC staining showing increased lung tumor cell apoptosis by PDLIM2 nanotherapy, immunotherapy, and further increase by their combination (n = 6). Scale bar in (A, D, F), 20 μm. Student's *t* test was performed (two tailed, unpaired) and data represent means ±□SEM. $^{**}p < 0.01$; ns, not statistically significant.

Figure 6. Combination of PDLIM2 nanotherapy, chemotherapy and immunotherapy shows great efficacy in lung cancer treatment. (A) IHC staining showing PD-L1 induction by chemotherapy but not PDLIM2 nanotherapy. (B) Schedule of lung cancer induction and treatment. (C) Urethane model showing high resistance of lung tumors to the chemo and αPD-1 combination therapy in lung epithelial specific PDLIM2 deletion mice (ΔSPC) (n ≥ 4). (D) IHC staining showing no MHC-I induction by chemotherapy, PD-1 immunotherapy or their combination (n = 6). (E) Tumor examination showing complete remission of all lung tumors in 60% of mice by combination of the three therapies (n ≥ 5). (F) IHC staining showing increased TILs by PDLIM2 nanotherapy in mice treated with anti-PD-1 and chemotherapeutic drugs (n = 6). (G) FACS analysis showing increased lung $CD8^+$ T-cell activation by PDLIM2 nanotherapy in mice treated with anti-PD-1 and chemotherapeutic drugs (n = 5). (H) No significant effect of PDLIM2 nanotherapy on the body weight of mice treated with anti-PD-1 and chemotherapeutic drugs (n = 5). Scale bar in (A and F), 20 μm. Student's *t* test was performed (two tailed, unpaired) and data represent means ±□SEM in (**c**-**g**). $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not statistically significant.

Figure 1

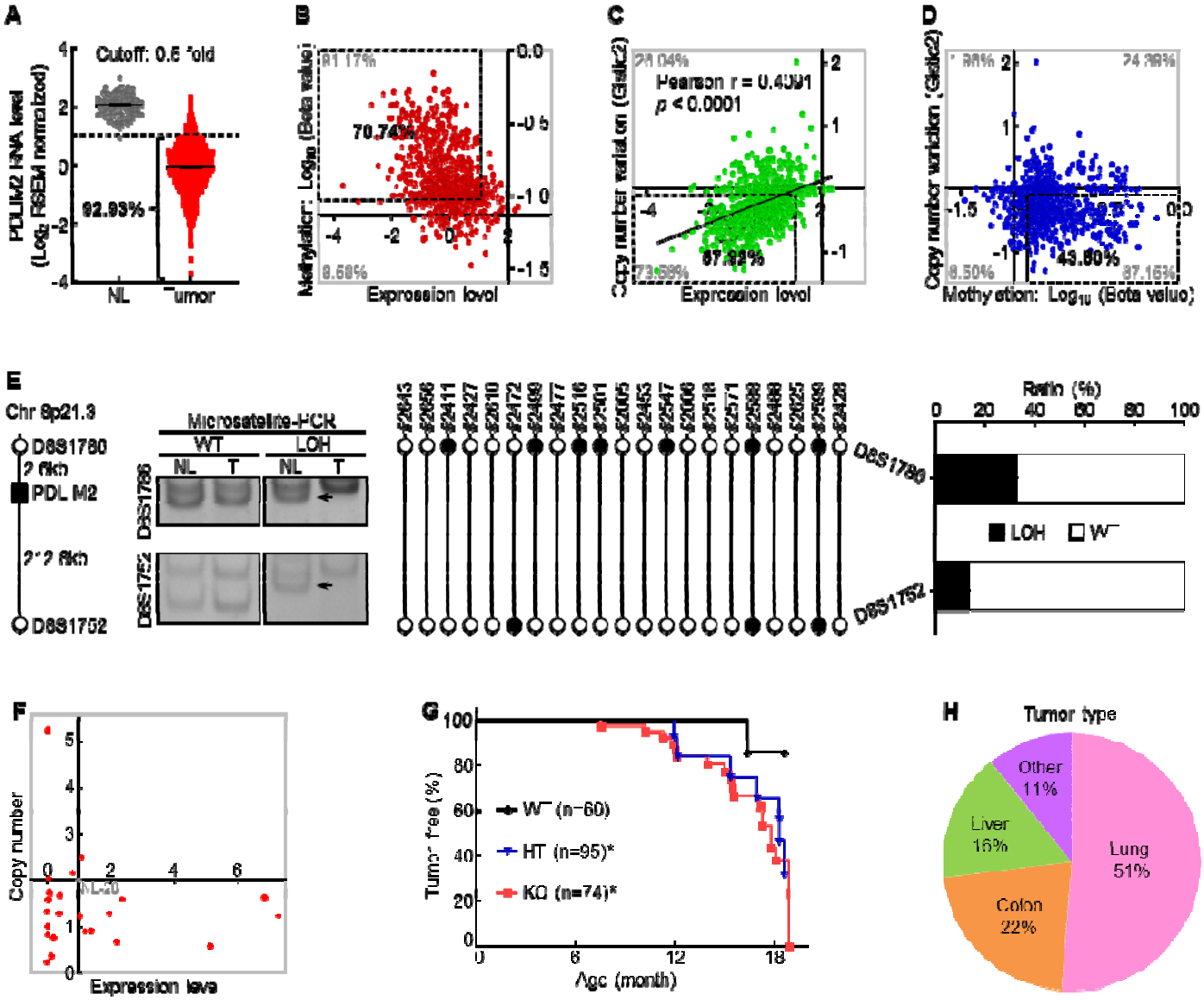

A
Cutoff: 0.6 fold
92.93%
NL
Tumor
B
70.74%
Expression level
C
Pearson r = 0.4091
p < 0.0001
Expression level
D
43.80%
E
Chr 8p21.3
D8S1780
PDL M2
D8S1752
Microsatellite-PCR
WT
LOH
NL
T
D8S1780
D8S1752
Ratio (%)
LOH
W⁻
F
Copy number
Expression level
G
W⁻ (n=60)
HT (n=95)*
KO (n=74)*
Age (month)
H
Tumor type
Other 11%
Liver 16%
Lung 51%
Colon 22%

Figure 2

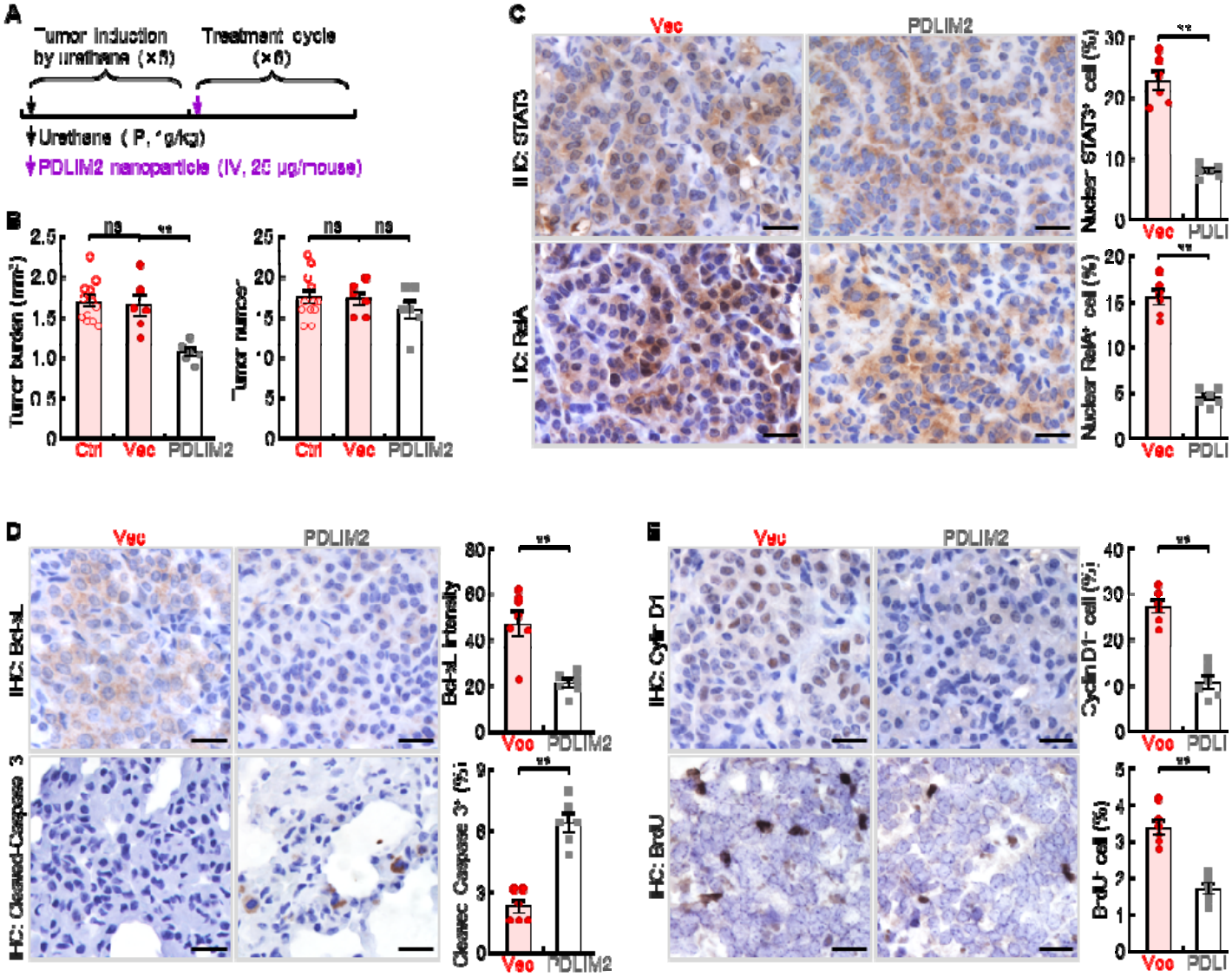

A
Tumor induction by urethane (×5)
Treatment cycle (×6)
Urethane (IP, 1 g/kg)
PDLIM2 nanoparticle (IV, 25 μg/mouse)
B
Tumor burden (mm²)
Tumor number
Ctrl
Vec
PDLIM2
ns
**
C
Vec
PDLIM2
IHC: STAT3
IHC: RelA
Nuclear STAT3⁺ cell (%)
Nuclear RelA⁺ cell (%)
D
IHC: Bcl-xL
IHC: Cleaved-Caspase 3
Bcl-xL Intensity
Cleaved Caspase 3⁺ (%)
E
IHC: Cyclin D1
IHC: BrdU
Cyclin D1⁺ cell (%)
BrdU⁺ cell (%)

Figure 3

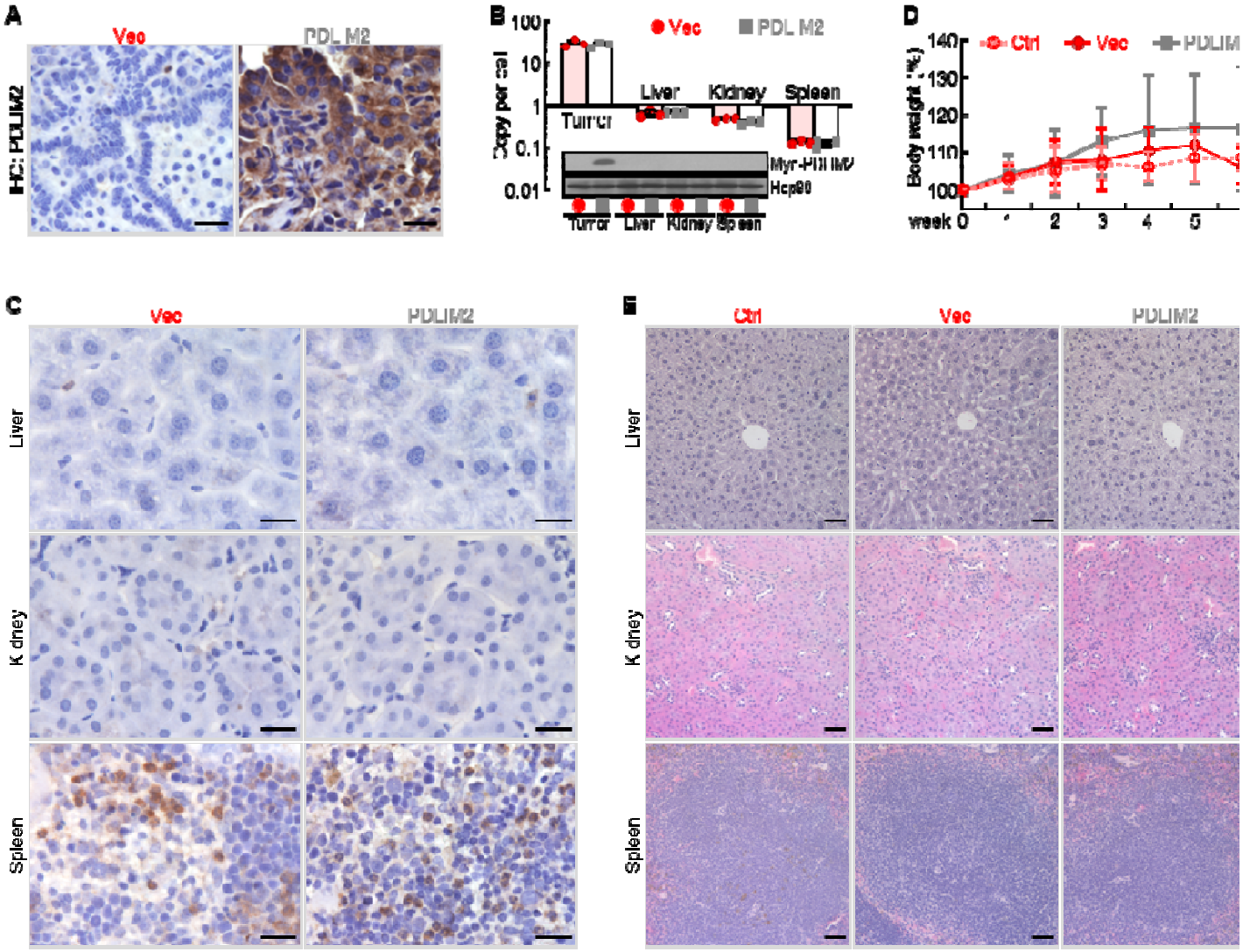

A
Vec
PDL M2
IHC: PDLIM2
B
Vec
PDL M2
Copy per cell
100
10
1
0.1
0.01
Tumor
Liver
Kidney
Spleen
Myr-PDLIM2
Hcp90
Tumor
Liver
Kidney
Spleen
D
Ctrl
Vec
PDLIM
Body weight (%)
140
130
120
110
100
week 0
2
3
4
5
C
Vec
PDLIM2
Liver
Kidney
Spleen
E
Ctrl
Vec
PDLIM2
Liver
Kidney
Spleen

Figure 4

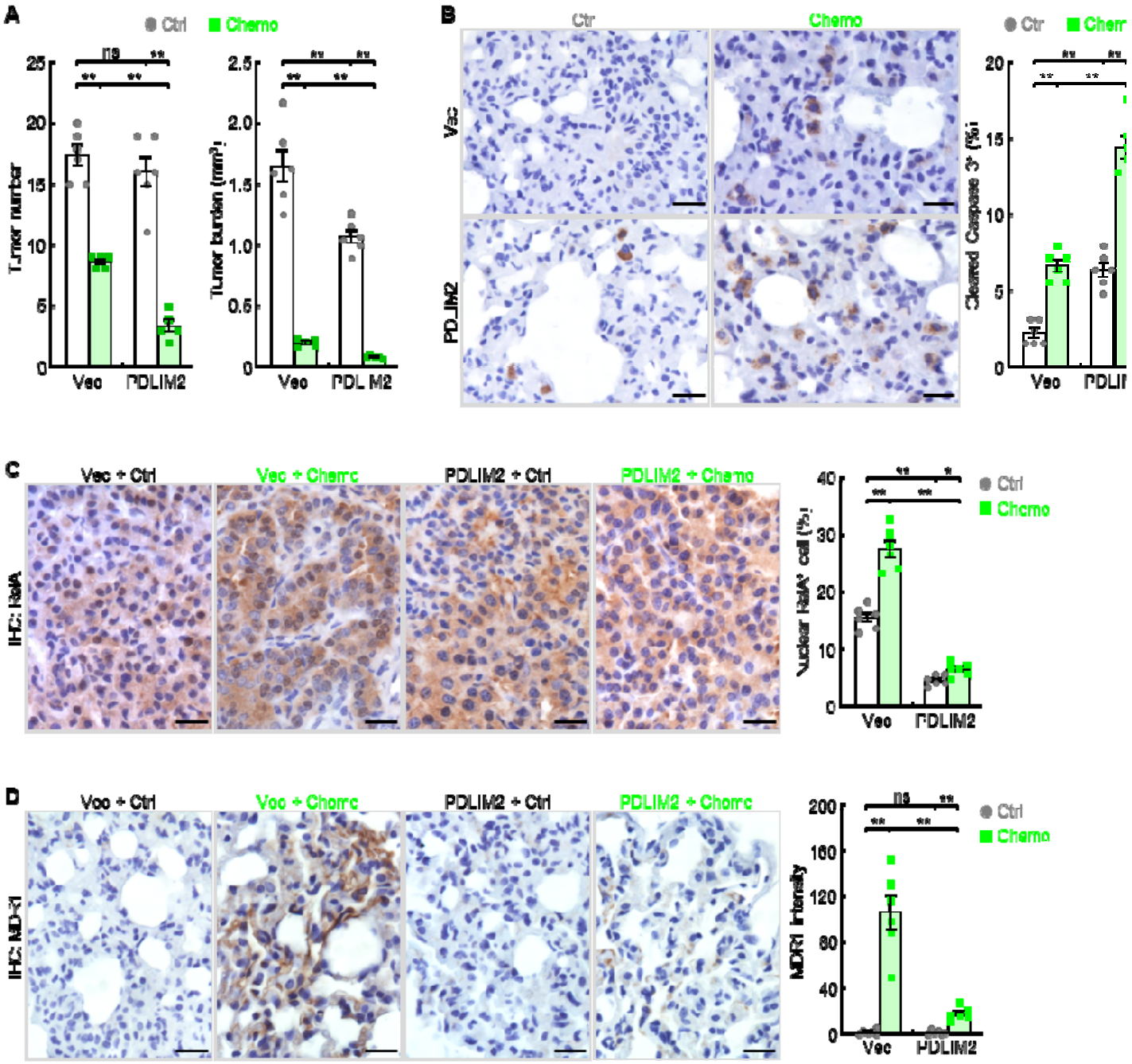

A
Ctrl
Chemo
Tumor number
Tumor burden (mm³)
Vec
PDLIM2
B
Ctr
Chemo
Vec
PDLIM2
Cleaved Caspase 3⁺ (%)
C
Vec + Ctrl
Vec + Chemo
PDLIM2 + Ctrl
PDLIM2 + Chemo
IHC: RelA
Nuclear RelA⁺ cell (%)
D
Vec + Ctrl
Vec + Chemo
PDLIM2 + Ctrl
PDLIM2 + Chemo
IHC: MDR1
MDR1 Intensity

Figure 5

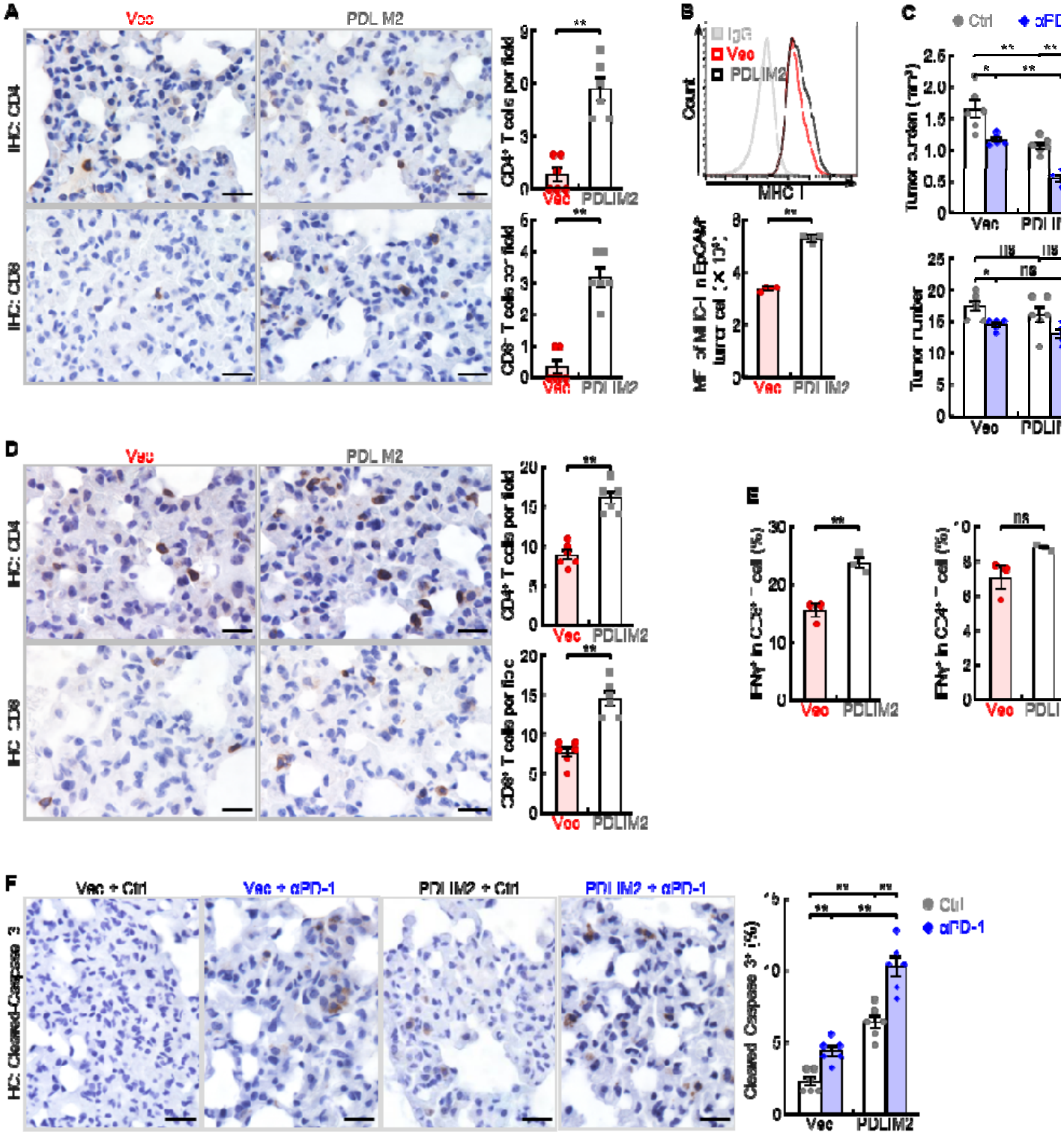
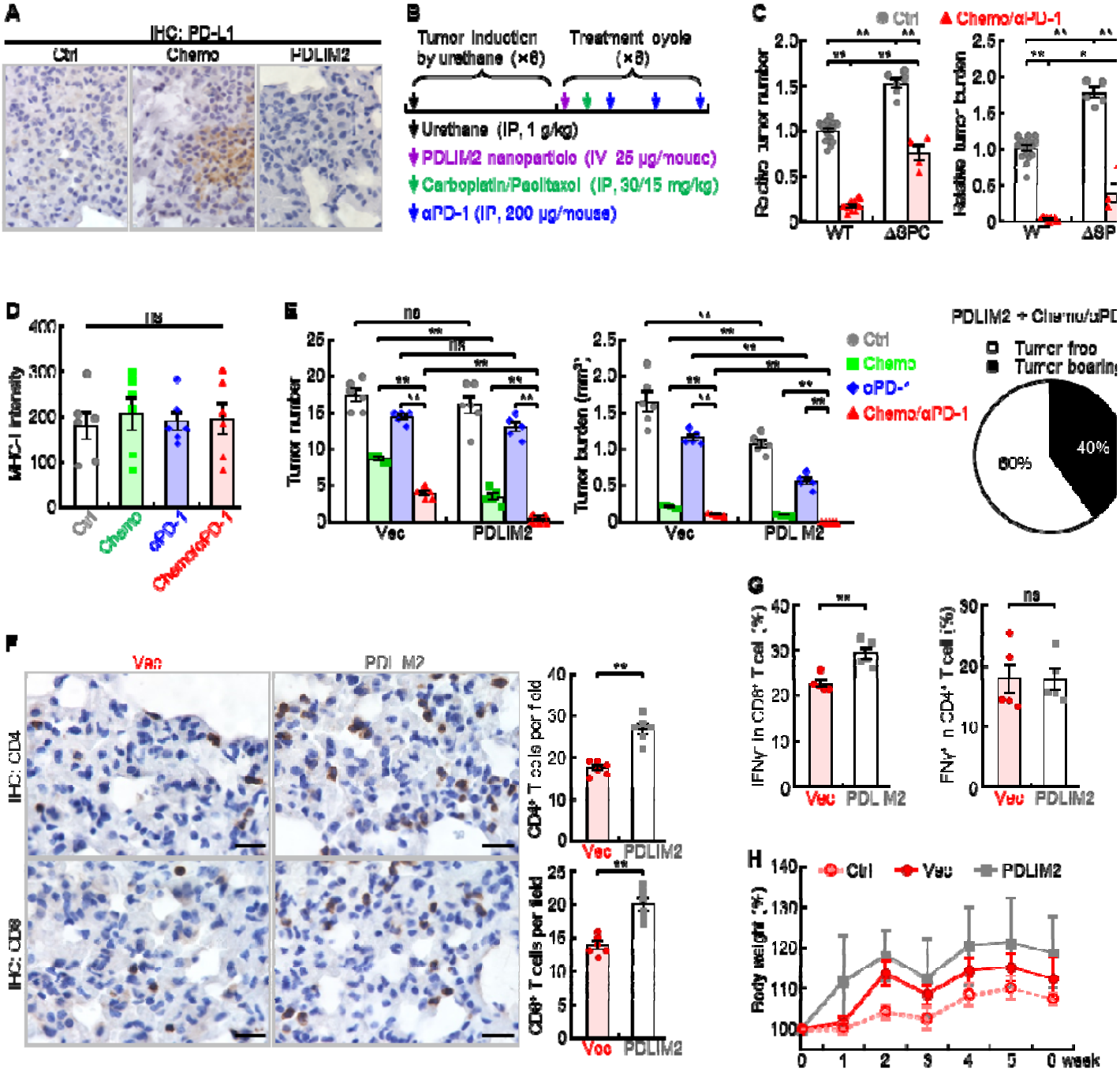

A
Vec
PDL M2
IHC: CD4
IHC: CD8
CD4+ T cells per field
CD8+ T cells per field
B
IgG
Vec
PDLIM2
Count
MHC I
C
Ctrl
αPD
Tumor number
D
E
IFNγ+ in CD8+ T cell (%)
IFNγ+ in CD4+ T cell (%)
F
Vec + Ctrl
Vec + αPD-1
PDLIM2 + Ctrl
PDLIM2 + αPD-1
IHC: Cleaved-Caspase 3
Cleaved Caspase 3+ (%)
αPD-1

Figure 6


A
IHC: PD-L1
Ctrl
Chemo
PDLIM2
B
Tumor induction by urethane (×6)
Treatment cycle (×8)
Urethane (IP, 1 g/kg)
PDLIM2 nanoparticle (IV, 25 μg/mouse)
Carboplatin/Paclitaxel (IP, 30/15 mg/kg)
αPD-1 (IP, 200 μg/mouse)
C
Ctrl
Chemo/αPD-1
Radio tumor number
Relative tumor burden
WT
ΔSPC
D
MHC-I intensity
Ctrl
Chemo
αPD-1
Chemo/αPD-1
E
Tumor number
Tumor burden (mm³)
Vec
PDLIM2
Ctrl
Chemo
αPD-1
Chemo/αPD-1
PDLIM2 + Chemo/αPD
Tumor free
Tumor bearing
60%
40%
F
Vec
PDLIM2
IHC: CD4
IHC: CD8
CD4⁺ T cells per field
CD8⁺ T cells per field
G
IFNγ⁺ in CD8⁺ T cell (%)
IFNγ⁺ in CD4⁺ T cell (%)
H
Ctrl
Vec
PDLIM2
Body weight (%)
week

682

Supplementary Table S1. Antibodies Used

Antibody	Company	Cat. No.	Dose	Usage
Anti-PDLIM2	Everest Biotech, Ramona, CA, USA	EB11878	1:400	IHC
Anti-Cleaved Caspase 3	Cell Signaling Technology, Danvers, MA, USA	9661	1:200	IHC
Anti-Bcl-xL	Cell Signaling Technology, Danvers, MA, USA	2764	1:2400	IHC
Anti-Cyclin D1	Santa Cruz Biotechnology, Dallas, TX, USA	sc-450	1:200	IHC
Anti-BrdU	Sigma-Aldrich, St. Louis, MO, USA	B2531	1:500	IHC
Anti-RelA	Cell Signaling Technology, Danvers, MA, USA	8242	1:500	IHC
Anti-STAT3	Cell Signaling Technology, Danvers, MA, USA	4904	1:1000	IHC
Anti-MDR1	Cell Signaling Technology, Danvers, MA, USA	13978	1:800	IHC
Anti-CD4	Abcam, Cambridge, MA, USA	ab183685	1:1000	IHC
Anti-CD8	Abcam, Cambridge, MA, USA	ab209775	1:1000	IHC
Anti-PD-L1	Cell Signaling Technology, Danvers, MA, USA	64988	1:200	IHC
Anti-MHC-I	Abcam, Cambridge, MA, USA	ab15681	1:200	IHC
Anti-mouse IgG Biotinylated	Vector Laboratories, Burlingame, CA, USA	BMK-2202	1:200	IHC
Goat anti-rat IgG Biotinylated	Vector Laboratories, Burlingame, CA, USA	BA9401	1:100	IHC
Rabbit anti-goat IgG Biotinylated	Santa Cruz Biotechnology, Dallas, TX, USA	sc-2774	1:200	IHC
Goat anti-rabbit IgG Biotinylated	Dako, Carpinteria, CA, USA	E0432	1:200	IHC
Anti-Hsp90	Santa Cruz Biotechnology, Dallas, TX, USA	sc-13119	1:1000	WB
Anti-c-Myc	Santa Cruz Biotechnology, Dallas, TX, USA	sc-40	1:1000	WB
Anti-CD16/CD32	eBioscience, San Diego, CA, USA	14-0161-85	1.0 μl per sample	FACS
Anti-CD45 FITC	Biolegend, San Diego, CA, USA	103107	0.5 μl per sample	FACS
Anti-EpCAM PE	eBioscience, San Diego, CA, USA	12-5791-82	0.625 μl per sample	FACS
Anti-MHC-I APC	eBioscience, San Diego, CA, USA	17-5998-82	1.25 μl per sample	FACS
Anti-CD3 PE	eBioscience, San Diego, CA, USA	12-0031-83	2.5 μl per sample	FACS
Anti-CD4 PE-Cy7	eBioscience, San Diego, CA, USA	25-0042-82	1.25 μl per sample	FACS
Anti-CD8a APC	eBioscience, San Diego, CA, USA	17-0081-83	0.625 μl per sample	FACS
Anti-IFNγ FITC	eBioscience, San Diego, CA, USA	11-7311-82	1 μl per sample	FACS
Anti-PD-1	BioXcell, West Lebanon, NH, USA	BE0273	200 μg/mouse/time	*in vivo* blockade

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Supplementary Table S2. Primers Used

Gene	Species	Accession number	Forward (5' to 3')	Reverse (5' to 3')	Usage
D8S1786	human		CGAAAGATTGAGACCCCAT	GTTTCCACACCGAAGCC	Microsatellite PCR
D8S1752	human		TCCTGGATCAGGCAGAAA	TCAGAGTTGGGTGAGCGA	Microsatellite PCR
LINE-1	human	M80343.1	AAAGCCGCTCAACTACATGG	TGCTTTGAATGCGTCCCAGAG	RT-PCR
PDLIM2	human	NG_030435.1	CAAAGCCAAGGACGCTGACC	GCTCTGGCGGATCTTGCTCT	RT-PCR
pdlim2	mouse	Mutant primer	GCTACCCGTGATATTGCTGAAGAG		Genotyping PCR
		WT primer	GTCTTCTCTTCCTCAGGCATGGCG		
		Common primer	GCCAACATTGCCCCAGAAGACTCC		
Amp-R	Plasmid		AACTTTATCCGCCTCCAT	GAAGCCATACCAAACGAC	Plasmid detection

Supplementary Table S3. PDLIM2 RNA level and copy number in human lung cancer cell lines

	PDLIM2 gene	
	Relative RNA level	Copy number
NL-20	1.000	2.000
CALU-6	0.010	1.021
H727	0.020	1.590
H460	0.001	1.341
H2122	0.042	2.038
H23	0.128	0.376
H358	7.260	1.244
SKLU-1	0.390	1.685
SW1573	0.010	0.842
CALU-1	1.950	1.293
128-88T	0.049	1.767
H1299	0.003	0.243
273T	0.815	2.172
H827	1.017	1.237
H1650	2.168	0.679
H3255	5.118	0.587
343T	1.365	0.919
CALU-3	0.377	1.290
H1435	1.198	0.915
H1793	6.833	1.637
H596	0.195	0.782
H838	0.080	1.740
H1838	2.360	1.603
A549	0.010	5.265
H1975	1.086	2.507

Supplement Figure S1

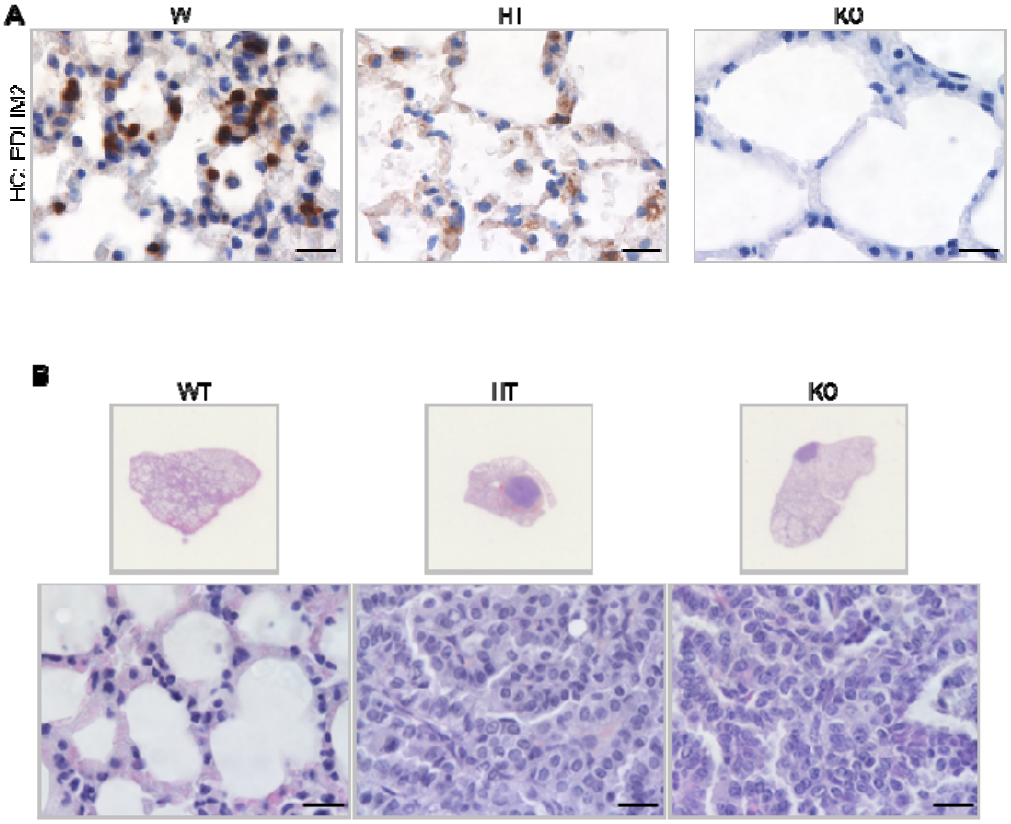

Figure S1. Mice with *Pdlim2* deletion develop spontaneous tumors, majorly lung tumors. A, IHC assays showing decreased and complete loss of PDLIM2 protein expression in the lungs of *Pdlim2*$^{+/-}$ (HT) and *Pdlim2*$^{-/-}$ (KO) mice, respectively. Scale bar, 20 µm. B, H&E staining of the lung tissues showing spontaneous lung tumors in *Pdlim2*$^{+/-}$ and *Pdlim2*$^{-/-}$ mice. Scale bar, 20 µm.

Supplement Figure S2

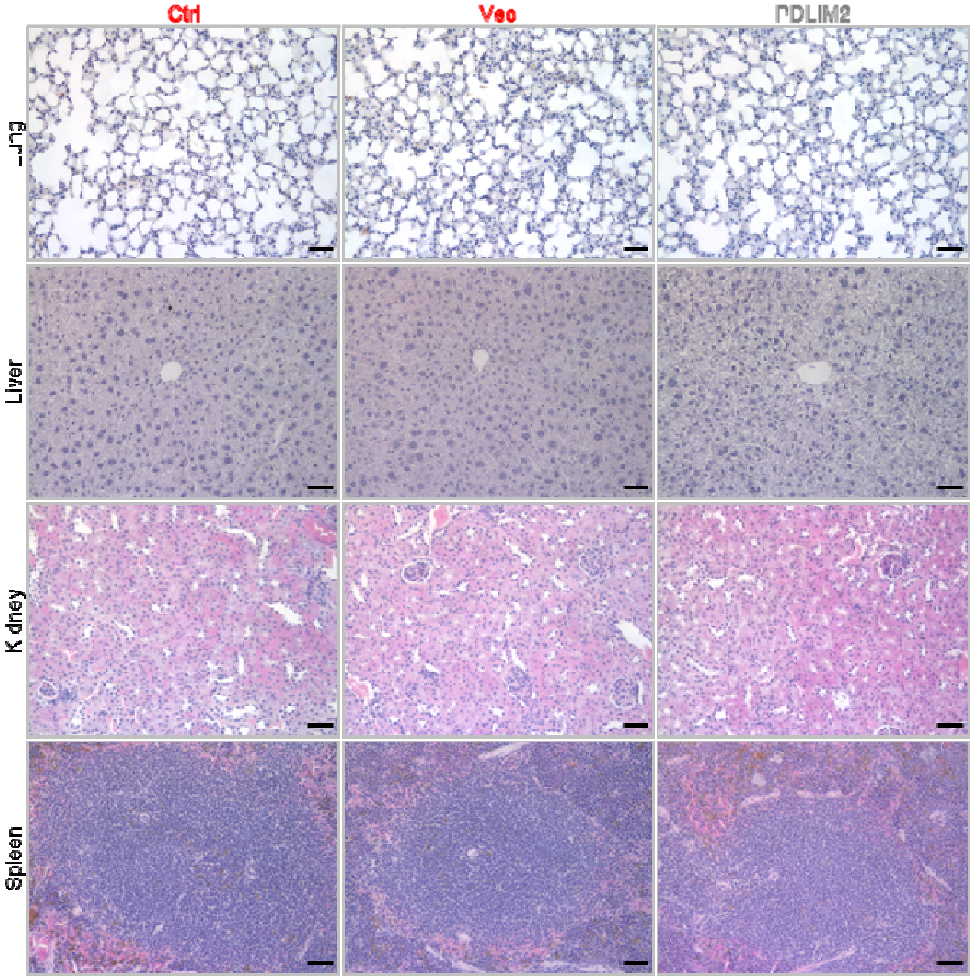

Figure S2. PDLIM2 nanotherapy causes no obvious pathological changes in major organs. H&E staining showing comparable patterns in lung, liver, kidney and spleen between Vec and PDLIM2 group in the context of combinational chemotherapy and PD-1 blockade immunotherapy (n = 5). Scale bar: 50 μm.

Supplement Figure S3

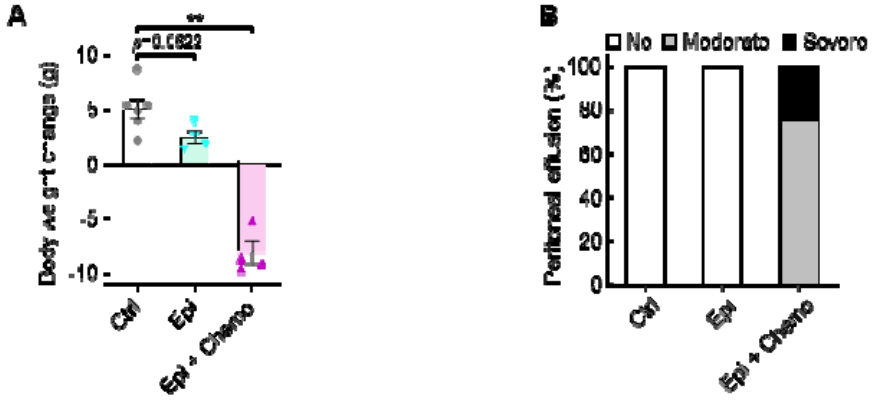

Figure S3. Epigenetic drugs cause body weight loss in mice with lung cancer. Mice were i.p. injected with urethane (1 g/kg) for 6 weeks to induce lung tumors, and then treated for 6 weeks with 5-aza-dC and MS-275 (Epi, i.p., 1 mg/kg each, twice per week), carboplatin and paclitaxel (Chemo, i.p., 30 mg/kg and 15 mg/kg, respectively, once per week), or their combinations. A, Body weight change between prior to the first treatment and the euthanasia endpoint. B, Percentage of mice with peritoneal effusion ($n \geq 4$).

Supplement Figure S4

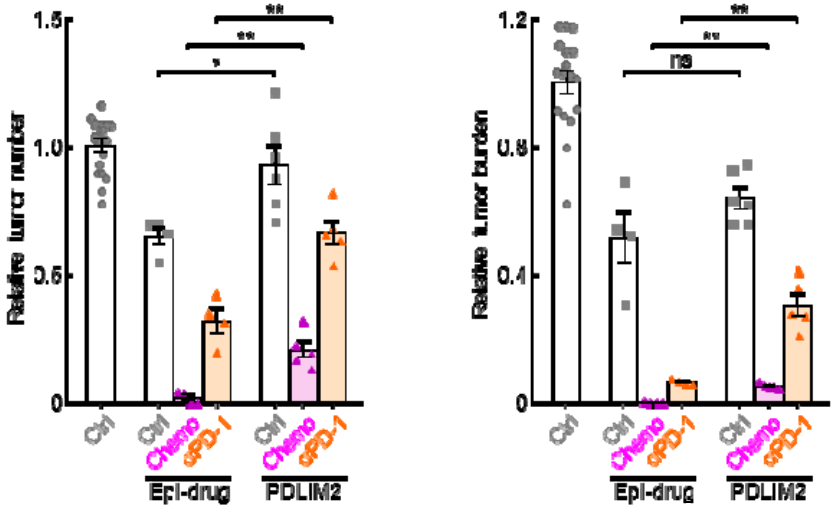

Figure S4. Epigenetic drugs show better efficacy than PDLIM2 nanotherapy in lung cancer treatment. Mice were i.p. injected with urethane (1 g/kg) for 6 weeks to induce lung tumors, and then treated with 5-aza-dC and MS-275 (Epi, i.p., 1 mg/kg each, twice per week), carboplatin and paclitaxel (Chemo, i.p., 30 mg/kg and 15 mg/kg, respectively, once per week), anti-PD-1 antibody (aPD-1, i.p., 200 μg/mouse, three times per week), PDLIM2-expression plasmids containing nanoparticles (i.v., 25 μg plasmid/mouse, once per week), or their combinations for 6 weeks before they were sacrificed for tumor examination ($n \geq 4$).