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Original Article

TRAIL receptor agonist TLY012 in combination with PD-1 inhibition promotes tumor regression in an immune-competent mouse model of pancreatic ductal adenocarcinoma

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) has an immunosuppressed, apoptosis-resistant phenotype. TLY012 is pegylated recombinant Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL), an orphan drug for chronic pancreatitis and systemic sclerosis. Innate immune TRAIL signaling suppresses cancer. We hypothesized that the combination of immune checkpoint-blocking anti-PD-1 antibody and TLY012 would have synergistic anti-tumor efficacy in immune-competent PDAC-bearing mice. PDAC tumor-bearing C57Bl/6 mice treated with 10 mg/kg anti-mouse PD-1 antibody twice weekly and 10 mg/kg TLY012 three times weekly had reduced tumor growth and tumor volume at 70 days compared to either drug alone (all $P < 0.005$). B-cell activating factor (BAFF), which promotes PDAC tumors, decreased to 44% of control mice with dual treatment at 7 days and remained decreased at 3 months. Long-term dual treatment showed the highest plasma levels of proinflammatory cytokines interferon-gamma (average 5.6 times control level, P=0.046), CCL5 (average 14.1 times control level, P=0.048), and interleukin-3 (IL-3, average 71.1 times control level, P=0.0053). Flow cytometry showed trends toward decreased circulating regulatory T cells, increased NK cells, and a higher proportion of CD8+ T cells within tumors in the dual treatment group. In summary, the combination of anti-PD-1 and TLY012 prevented the growth of PDAC in an immunocompetent mouse model while increasing tumor-infiltrating CD8+ T cells, decreasing circulating T-regulatory cells and altering plasma cytokine expression of CCL5, interferon-gamma, and IL-3 to promote proinflammatory, antitumor effects. Combining TLY012 and anti-mouse PD-1 modifies immune cell and cytokine levels to induce a more proinflammatory immune environment that contributes to decreased PDAC tumor growth.

Keywords: TLY012, TRAIL, BAFF, CCL5, IFN-gamma, IL3, inflammation, cancer

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains a deadly disease with little progress in long-term survival over the last several decades [1-5]. Much work has been done to characterize molecular alterations and drivers in pancreatic cancer including among different ethnic groups, early onset pancreatic cancer, and treatment effects on the genomic landscape [6-13]. Some of the key driver alterations include mutations in KRAS, p53, p16, SMAD4, KDM6A, ARID1A, and BRCA2, signatures of DNA damage and repair deficiency, mismatch repair deficiency as well as amplified genes such as ERBB2, MET, PIK3CA among others [6, 7, 11, 12]. While much progress has been made with immunotherapy across tumor types, and much understanding

of the biology of pancreatic cancer, there has been little impactful progress on advanced PDAC in part due to an immunosuppressed, apoptosis-resistant phenotype [14-23].

The TNF-Related Apoptosis-Inducing Ligand (TRAIL) is part of the host immune system that suppresses transformed cells, virally infected cells as well as cancer and its metastases [24-35]. Nearly 3 decades ago, the discovery of pro-apoptotic TRAIL Death Receptor DR5 as a direct transcriptional target of the p53 tumor suppressor gene directly linked the innate immune system to the host response to suppress tumorigenesis [36-42]. Deletion of TRAIL receptor DR5 in mice led to apoptotic resistance in vivo as well as tumorigenesis [43, 44]. It was later recognized that the TRAIL gene is also a p53 target gene and subsequent work identified TRAIL-inducing compound #10 (TIC10; also known as ONC201) [45, 46]. We previously reported that the combination of TRAIL or TRAIL receptor DR5 agonist antibodies and ONC201 has particularly potent anti-tumor effects *in vivo* across cancer types including pancreatic cancer [47-50]. We have also previously reported a TRAIL-inducing micro-RNA that we have not, as of the date of publication of this manuscript, translated to *in vivo* studies or to the treatment of patients [51].

The TRAIL pathway influences both inflammation and tumorigenesis as well as fibrosis, all relevant to PDAC and its potential novel therapeutics [44, 52-55]. TLY012 is pegylated recombinant Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) [55, 56].

In the present studies, we explore the hypothesis that TLY012's modulation of the tumor microenvironment has the potential for synergistic effects when combined with immune checkpoint blockade. We demonstrate potent anti-tumor effects of TLY012 combined with anti-mouse PD-1 and show immune alterations including in B- and T-cell immunity as well as tumor-promoting cytokines. Our results prompt further preclinical and clinical studies evaluating the novel treatment combination of TLY012 and anti-PD1.

Materials and methods

Cell culture

Cell lines used for this study were acquired from the American Type Culture Collection (ATCC), unless otherwise indicated. All pancreatic cancer cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Human Foreskin Fibroblasts (HFF) were grown in DMEM with 15% FBS. rhTRAIL was generated in-house using a protocol previously developed by our lab and detailed in *Kim et al.* TLY012 was provided by Theraly Fibrosis, Inc. TLY012 was diluted in sterile phosphate buffered saline (PBS) for *in vitro* experiments.

Cell viability assay

To assess cell viability, cells were plated overnight in 96-well plates at a density of 1.0×10^4 cells/well. All cells were plated in triplicates. After 72 hours, the media in the wells was replaced with either fresh media (controls) or with media containing various doses of rhTRAIL or TLY012. After 4 hours of incubation, cells were treated with Cell-Titer Glo (Promega) and imaged to assess cell viability. Synergy and combination indices were determined using Compusyn, which uses the Chou-Talalay method for determining synergy.

Cytokine profiling

Murine plasma samples were collected at 7 days and 3 months post-treatment with vehicle control, anti-PD-1, TLY012, or the combination of anti-PD-1 + TLY012. Samples were analyzed using an R&D systems Murine Premixed Multi-Analyte Kit (R&D Systems, Inc., Minneapolis, MN, USA) and a Luminex 200 Instrument (LX200-XPON-RUO, Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. Samples were analyzed in duplicate and average sample values are reported in pg/mL.

Flow cytometric analysis

Murine spleens and tumors were collected 3 months post-treatment. Flow cytometry viability staining was conducted by suspending murine spleen and tumor single cell suspensions in Zombie Violet solution (BioLegend, San Diego, CA, USA) according to manufacturer instructions for 30 minutes at room temperature. Staining for membrane surface proteins was conducted using conjugated primary antibodies for 1 hour on ice, according to manufacturer instructions. The following antibodies were used for the described T cell profiling

experiments: CD45 Monoclonal Antibody (30-F11), eVolve™ 605, eBioscience™, Catalog # 83-0451-42; BD Pharmingen™ APC-Cy™7 Rat Anti-Mouse CD3 Molecular Complex, Clone 17A2 (RUO), Catalog # 560590; CD4 Monoclonal Antibody (RM4-5), PE-Cyanine7, eBioscience™, Catalog # 25-0042-82; BD Pharmingen™ PE Rat Anti-Mouse CD8a, Clone 53-6.7 (RUO), Catalog # 553032; Invitrogen CD69 Monoclonal Antibody (H1.2F3), FITC, eBioscience™, Catalog # 11-0691-81; Invitrogen FOXP3 Monoclonal Antibody (FJK-16s), APC, eBioscience™, Catalog # 17-5773-82. The following antibodies were used for the described NK cell profiling experiments: CD45 Monoclonal Antibody (30-F11), eVolve™ 605, eBioscience™, Catalog # 83-0451-42; BD Pharmingen™ PE Rat Anti-Mouse CD3 Molecular Complex, Clone 17A2 (RUO), Catalog # 555275; Invitrogen NK1.1 Monoclonal Antibody (PK136), APC, eBioscience™, Catalog # 17-5941-82; APC/Cyanine7 anti-mouse/human CD11b Antibody, Clone M1/70, Catalog # 101226; Invitrogen CD27 Monoclonal Antibody (LG.7F9), FITC, eBioscience™, Catalog # 11-0271-82; Invitrogen KLRG1 Monoclonal Antibody (2F1), PE-Cyanine7, eBioscience™, Catalog # 25-5893-82. Cells were fixed using IC Fixation Buffer (eBioscience™, San Diego, CA, USA) for 30 minutes according to manufacturer instructions. Cells were resuspended in Flow Cytometry Staining Buffer (R&D Systems, Minneapolis, MN, USA) and were analyzed using a BD Biosciences LSR II and FlowJo version 10.1 (FlowJo, Ashland, OR, USA).

Natural killer cell immunophenotyping

The NK cell flow cytometry panel included the following directly-conjugated primary antibodies: CD45 Monoclonal Antibody (30-F11), eVolve™ 605, eBioscience™, Catalog # 83-0451-42; BD Pharmingen™ PE Rat Anti-Mouse CD3 Molecular Complex, Clone 17A2 (RUO), Catalog # 555275; Invitrogen NK1.1 Monoclonal Antibody (PK136), APC, eBioscience™, Catalog # 17-5941-82; APC/Cyanine7 anti-mouse/human CD11b Antibody, Clone M1/70, Catalog # 101226; Invitrogen CD27 Monoclonal Antibody (LG.7F9), FITC, eBioscience™, Catalog # 11-0271-82; Invitrogen KLRG1 Monoclonal Antibody (2F1), PE-Cyanine7, eBioscience™, Catalog # 25-5893-82. Gating strategies are as follows: NK cell: live/CD45/CD3-/NK1.1+; Mature NK cell: live/CD45/CD3-/NK1.1+/KRLG1+; Activated NK cell: live/CD45/CD3-/NK1.1+/CD11b+; NK cell subset 1: live/CD45/CD3-/NK1.1+/CD11blowCD27low; NK cell subset 2: live/CD45/CD3-/NK1.1+/CD11blowCD27high; NK cell subset 3: live/CD45/CD3-/NK1.1+/CD11bhighCD27high; NK cell subset 4: live/CD45/CD3-/NK1.1+/CD11bhighCD27low.

T cell immunophenotyping

The T cell flow cytometry panel included the following directly-conjugated primary antibodies: CD45 Monoclonal Antibody (30-F11), eVolve™ 605, eBioscience™, Catalog # 83-0451-42; BD Pharmingen™ APC-Cy™7 Rat Anti-Mouse CD3 Molecular Complex, Clone 17A2 (RUO), Catalog # 560590; CD4 Monoclonal Antibody (RM4-5), PE-Cyanine7, eBioscience™, Catalog # 25-0042-82; BD Pharmingen™ PE Rat Anti-Mouse CD8a, Clone 53-6.7 (RUO), Catalog # 553032; Invitrogen CD69 Monoclonal Antibody (H1.2F3), FITC, eBioscience™, Catalog # 11-0691-81; Invitrogen FOXP3 Monoclonal Antibody (FJK-16s), APC, eBioscience™, Catalog # 17-5773-82. Gating strategies are as follows: CD4+ T cell: live/CD45+/CD3+/CD4+/FOXP3-; CD8+ T cell: live/CD45+/CD3+/CD8+; Treg: live/CD45+/CD3+/CD4+/FOXP3+; Activated CD8+ T cell: live/CD45+/CD3+/CD8+/CD69+.

In vivo tumor xenograft studies

All *in vivo* studies conducted for this manuscript were approved by the Brown University IACUC. For *in vivo* tumor xenograft studies, we used female C57Bl/6 mice. Mice were aged 5-8 weeks at the time of tumor inoculation. Cells were mixed in a 50:50 Matrigel (Corning): PBS solution and mixed at various dilutions. The total inoculation volume was 200 µL, irrespective of the tumor model or the number of cells inoculated. The vehicle is a solution of 20% Cremophor EL (Sigma-Aldrich), 70% PBS, and 10% DMSO. rhTRAIL was administered through intravenous tail vein injections. TLY012 was administered via intraperitoneal injections.

Tumor volume calculations

Measurements were taken using Vernier calipers. The equation we employed for calculating tumor volume is: Volume = $(Width^2 * Length)/2$.

Treatment was initiated once the tumors reached an optimal volume between 100-150 mm^3.

Statistical analysis

The statistical significance of differences between pairs was determined using unpaired Student's t-tests. The statistical significance between groups was determined using a One-way ANOVA followed by a post-hoc Tukey's multiple comparisons test. The minimal level of significance was $P < 0.05$. Following symbols * and ** represent, $P < 0.05$ and $P < 0.01$, respectively.

Results

Potent anti-tumor effects in PDAC-implanted syngeneic C57Bl/6 mice treated with anti-mouse PD-1 and TLY012

TLY012, a novel TRAIL formulation, activates the host innate immune system through the TRAIL pathway and targets fibrosis within the tumor microenvironment. These properties including removal of an immune suppressive microenvironment are well suited for synergy with other agents such as immune checkpoint therapy that may have improved penetration and efficacy in the tumor microenvironment.

We set up a mouse model treatment scheme (**Figure 1A**) to test the hypothesis that combination of immune checkpoint-blocking anti-PD-1 antibody and TLY012 would have synergistic anti-tumor efficacy in immune-competent PDAC-bearing mice.

We used immune competent C57/Bl6 mice as the host for our experiments where 50,000 KPC-Luc PDAC cells were implanted subcutaneously. As shown in **Figure 1A**, we included cohorts of mice for short-term analysis including analysis of tumor-infiltrating lymphocytes (TIL) as well as systemic immune analysis, cytokine analysis, immunohistochemical biomarker analysis and toxicity analysis. Cohort sizes are indicated in **Figure 1A**. In addition, there were mice allocated to a long-term cohort for similar analyses. Treatments included "adjuvant control" (6 mice for short-term and 9 mice for long-term), TLY012 at 10 mg/kg 3 times per week by intraperitoneal (IP) injection (4 mice for short-term and 6 mice for long term), anti-mouse PD1 at 10 mg/kg 2 times per week by IP injection (4 mice for short-term and 6 mice for long term), and the combination of TLY012 plus anti-PD1 therapy (4 mice for short-term and 6 mice for long term).

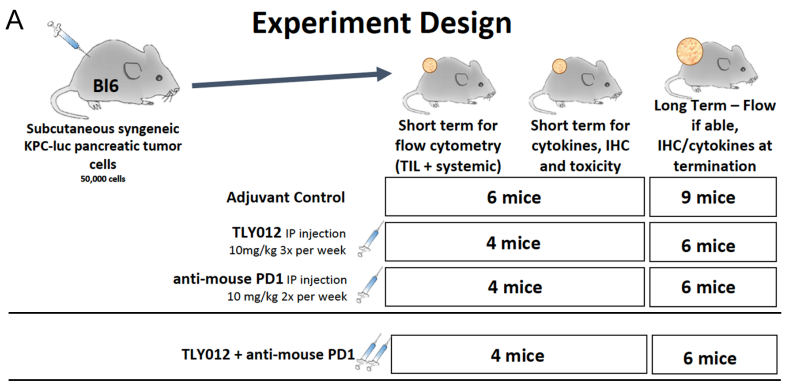
The result of the long-term *in vivo* study demonstrates potent anti-tumor effects in PDAC-implanted syngeneic C57Bl/6 mice treated with anti-mouse PD-1 and TLY012 (**Figure 1B**). KPC-Luc tumors grew over 20 days at which point treatments were initiated as indicated in **Figure 1A**. Short-term analysis of immune cells (TILs and systemically), cytokines and immunohistochemistry as well as toxicity analysis were performed at 7 days after treatment initiation for the different cohorts of mice. Long-term analysis was performed as indicated after continued treatments of the long-term treatment cohorts as indicated in **Figure 1A**. The results demonstrate statistically significant reduction in tumor volumes and tumor weights in the monotherapy cohorts (TLY012 at 10 mg/kg 3 times per week IP or anti-mouse PD1 at 10 mg/kg 2 times per week by IP injection) and potent suppression of tumors with combination therapy with TLY012 plus anti-PD1 therapy over the course of the experiment as shown in **Figure 1B**.

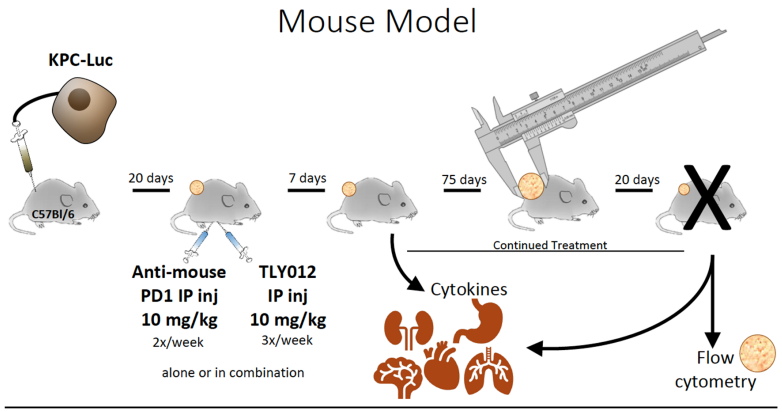
Available tumors at the end of the experiment were photographed (**Figure 1C**) and correspond to the results shown in **Figure 1B**. The mice tolerated the treatments well with no reduction in mouse weights (**Figure 1D**).

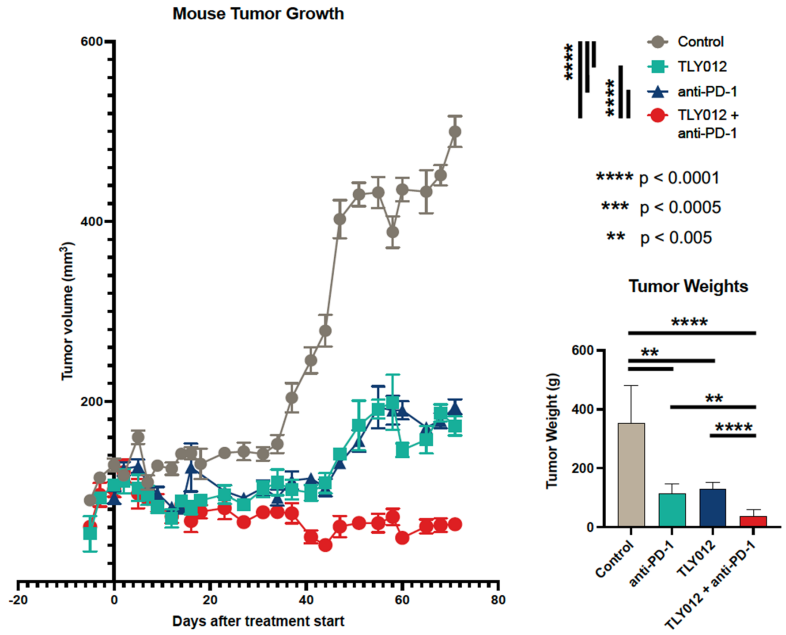
Decreased circulating regulatory T-cells, increased NK-cells, and a higher proportion of CD8+ T cells within tumors after dual treatment with anti-mouse PD-1 and TLY012

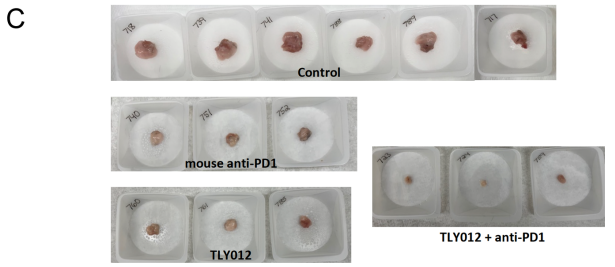
We performed flow cytometric analyses of spleen and tumor T-cells and NK-cells to examine the effects of individual anti-mouse PD-1 or TLY012, as well as the dual anti-mouse PD-1 and TLY012 treatment group in treated syngeneic C57Bl/6 KPC-Luc PDAC tumor-bearing mice (**Figure 2**).

We observed a reduction in splenic T-regulatory as cytotoxic CD8+ T-cells (**Figure 2A**) along with a relative enrichment in analyzed tumors of cytotoxic CD8+ T-cells in combination anti-mouse PD-1 and TLY012-treated tumors (**Figure 2B**) in the syngeneic KPC PDAC model. We observed enrichment of immature NK cells in the spleen of combo treated mice (**Figure 2C**)


A
Experiment Design
B16
Subcutaneous syngeneic KPC-luc pancreatic tumor cells
50,000 cells
Short term for flow cytometry (TIL + systemic)
Short term for cytokines, IHC and toxicity
Long Term – Flow if able, IHC/cytokines at termination
Adjuvant Control
6 mice
9 mice
TLY012 IP injection 10mg/kg 3x per week
4 mice
6 mice
anti-mouse PD1 IP injection 10 mg/kg 2x per week
4 mice
6 mice
TLY012 + anti-mouse PD1
4 mice
6 mice


Mouse Model
KPC-Luc
C57Bl/6
20 days
7 days
75 days
20 days
Continued Treatment
Anti-mouse PD1 IP inj 10 mg/kg 2x/week
TLY012 IP inj 10 mg/kg 3x/week
alone or in combination
Cytokines
Flow cytometry


B
Mouse Tumor Growth
Tumor volume (mm³)
Days after treatment start
Control
TLY012
anti-PD-1
TLY012 + anti-PD-1
**** p < 0.0001
*** p < 0.0005
** p < 0.005
Tumor Weights
Tumor Weight (g)
Control
anti-PD-1
TLY012
TLY012 + anti-PD-1


C
Control
mouse anti-PD1
TLY012
TLY012 + anti-PD1

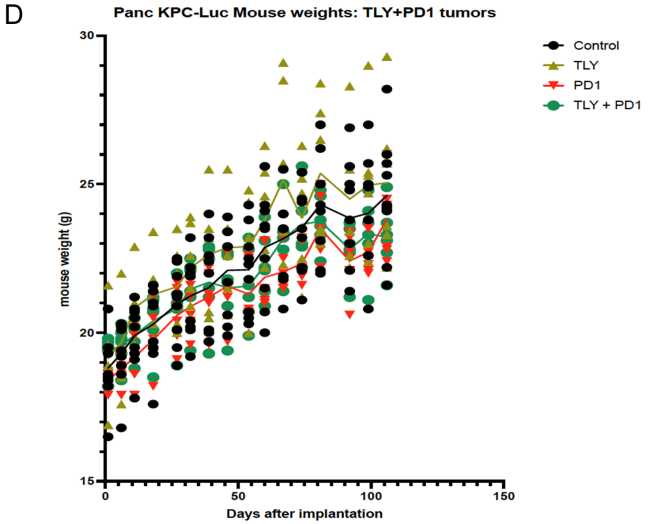
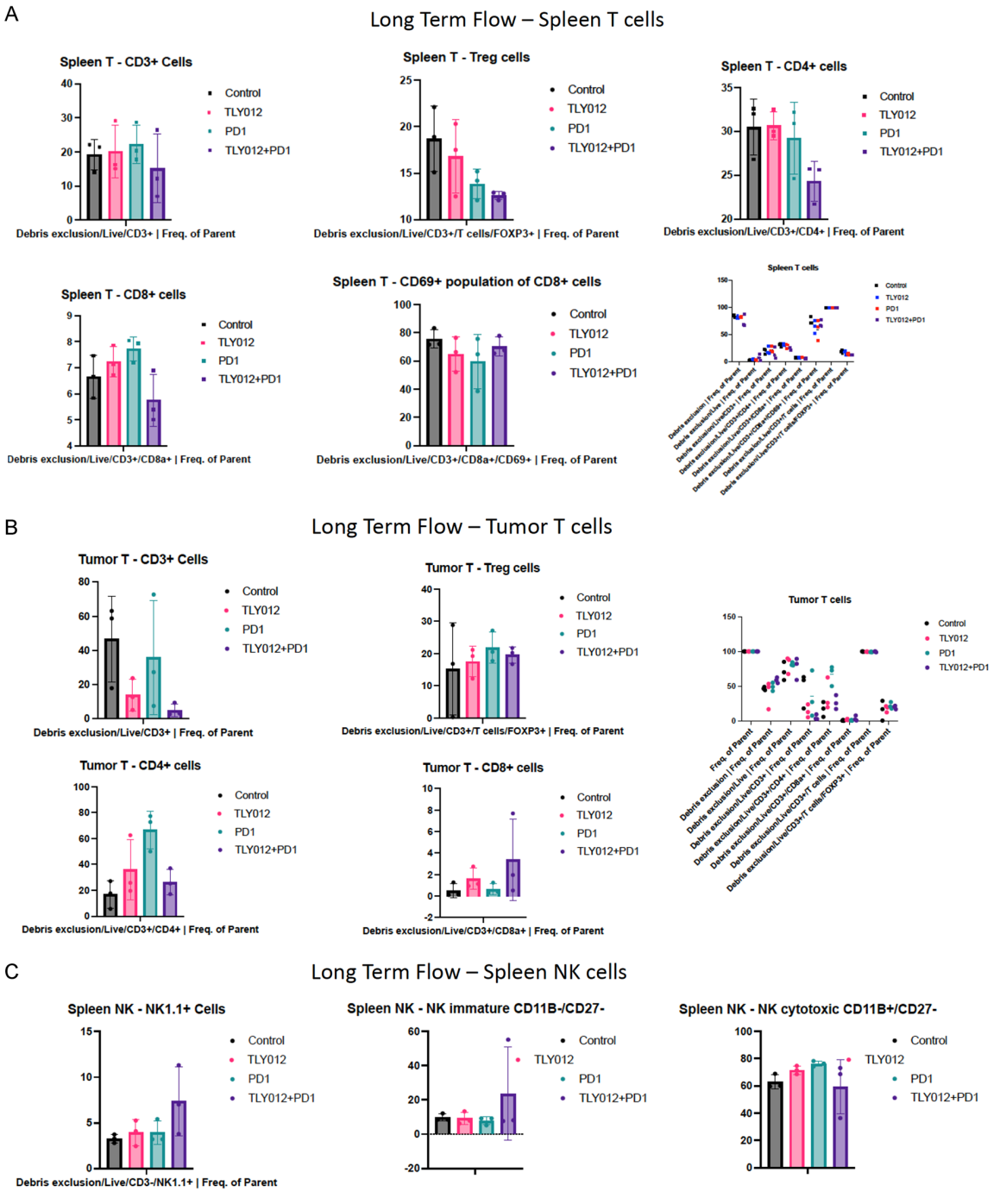

D
Panc KPC-Luc Mouse weights: TLY+PD1 tumors
mouse weight (g)
Days after implantation
Control
TLY
PD1
TLY + PD1

Figure 1. Syngeneic mice with PDAC treated with anti-mouse PD-1 and TLY012 have delayed tumor growth and reduced tumor volume. A. *In vivo* PDAC mouse model and treatment schema for anti-tumor efficacy, biomarker, and immune analysis. The number of mice used for short-term as well as long-term treatments is as indicated for each cohort. B. Syngeneic mice with PDAC treated with both anti-mouse PD-1 and TLY012 resulted in slower tumor growth and reduced tumor volume at 70 days compared to either drug alone (all $P < 0.005$). Tumor weights are shown in the graph in the lower right for the respective treatment groups. C. Images of available tumors at the end of the experiment are shown. D. Weights of mice in different treatment groups over the course of the experiment.



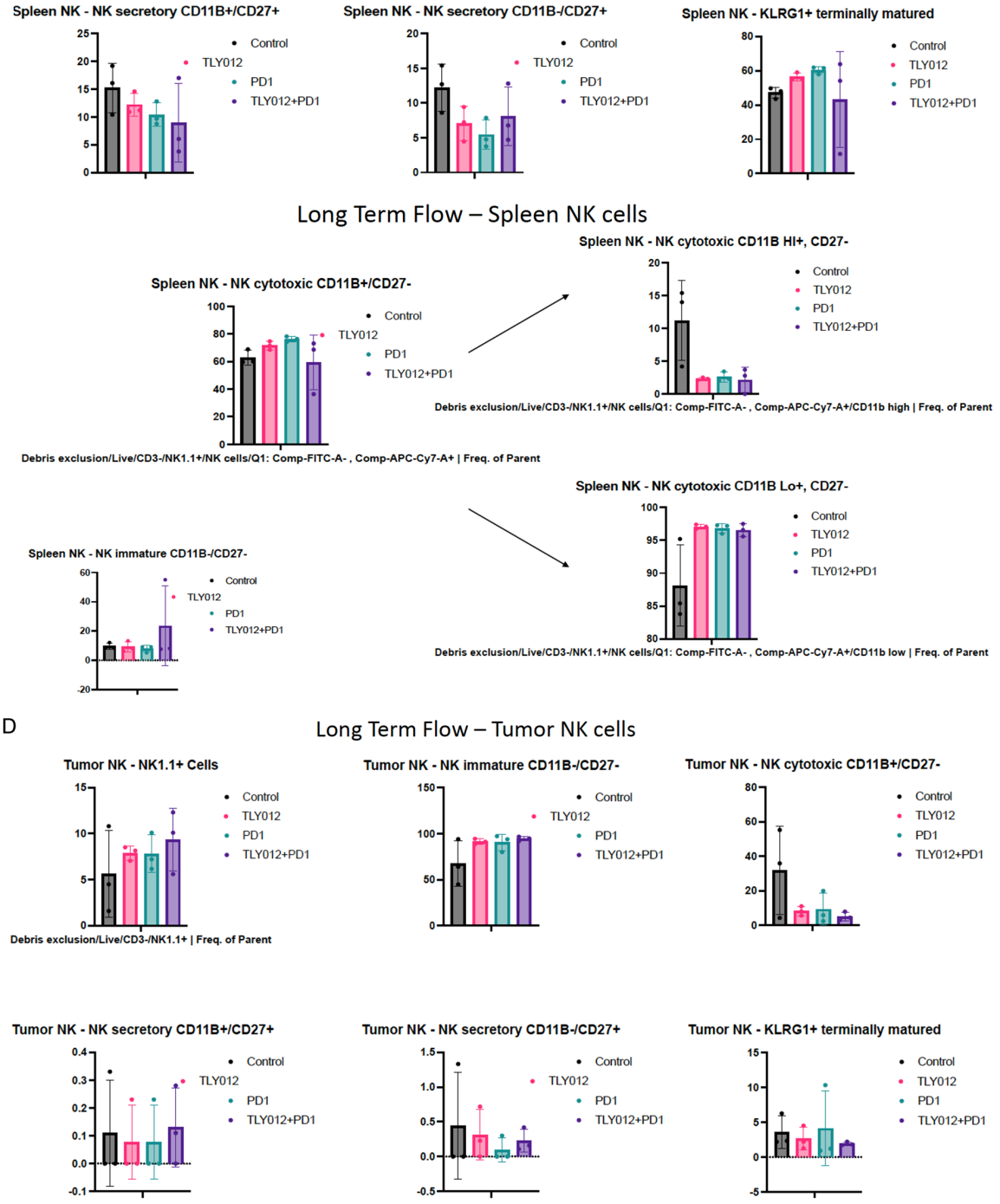


Figure 2. Decreased circulating regulatory T-cells, increased NK-cells, and a higher proportion of CD8+ T cells within tumors after dual treatment with anti-mouse PD-1 and TLY012. A. Flow cytometric immune analysis of spleen T cells following 3 month treatment with TLY012, anti-PD1 or the combination of TLY012 and anti-PD1 in a syngeneic pancreatic cancer mouse model (KPC-Luc). B. Flow cytometric immune analysis of tumor T following treatment with TLY012, anti-PD1 or the combination of TLY012 and anti-PD1 in a syngeneic pancreatic cancer mouse model (KPC-Luc). C. Flow cytometric immune analysis of spleen NK cells following treatment with TLY012, anti-PD1 or the combination of TLY012 and anti-PD1 in a syngeneic pancreatic cancer mouse model (KPC-Luc). D. Flow cytometric immune analysis of tumor NK cells following treatment with TLY012, anti-PD1 or the combination of TLY012 and anti-PD1 in a syngeneic pancreatic cancer mouse model (KPC-Luc).

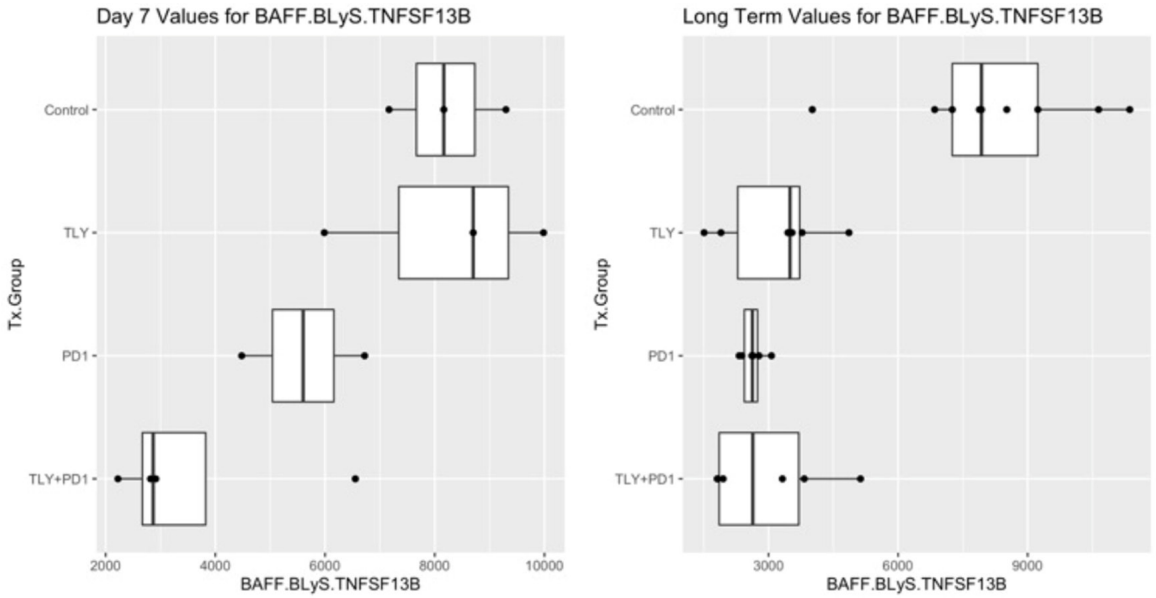


Figure 3. Reduced tumor-promoting B-cell activating factor (BAFF) levels in anti-PD1 treated and dual TLY012 plus anti-PD1 treated mice at 7 days and in single and dual treated groups at three months. Day 7 murine plasma cytokine levels are in the left panel and the levels at 3 months are shown in the right panel for the different treatment groups as indicated. The numbers shown for plasma BAFF levels on the X-axes are in pg/mL.

and similarly the tumors of dual anti-mouse PD-1 and TLY012 treated PDAC tumor-bearing C57Bl/6 mice showed enrichment with immature NK cells (**Figure 2D**). Thus, the combination of TLY012 plus anti-PD1 enriches treated tumors with cytotoxic CD8+ T-cells as well as immature NK cells. These findings are consistent with the observed synergistic anti-tumor efficacy of the combination therapy *in vivo*.

B-cell activating factor (BAFF), which correlates with tumor progression in PDAC, was reduced in treatment groups at 7 days and 3 months

B-cell activating factor (BAFF) correlates with tumor progression in PDAC [57]. Plasma BAFF concentrations were reduced in anti-PD1-treated and dual TLY012 plus anti-PD1-treated mice at 7 days and was reduced in single and dual-treated groups after three months (**Figure 3**). Reduced BAFF levels are consistent with the observed anti-PDAC effects of treatment with TLY012 plus anti-PD1.

Long-term plasma cytokine analysis following treatment with TLY012, anti-PD1 or the combination of TLY012 and anti-PD1 in syngeneic KPC PDAC mouse model

We assessed plasma cytokine levels at 3 months in the TLY012, anti-PD1 or combination treatment groups versus control mice (**Figure 4**). A few proinflammatory cytokines including interferon-gamma and CCL5 were elevated while VEGFR2 trended towards reduction in the long-term dual treatment group (**Figures 4**, **5**). These results are consistent with the observed anti-PDAC effects of treatment with TLY012 plus anti-PD1.

Discussion

We report potent anti-tumor effects following long-term treatment for 3 months with the combination of innate immune anti-fibrotic TLY012 and immune checkpoint blocker anti-mouse PD-1 in a KPC PDAC syngeneic C57Bl/6 mouse model. The treatments were associated with changes in immune cell populations in spleens and tumors and plasma cytokine levels to induce a more proinflammatory immune environment that correlates to decreased PDAC tumor growth.

A limitation of our study is that the tumors were implanted subcutaneously and not orthotopically which could influence the results due to differences in the local microenvironment. Clearly, this would need to be further addressed in future studies.

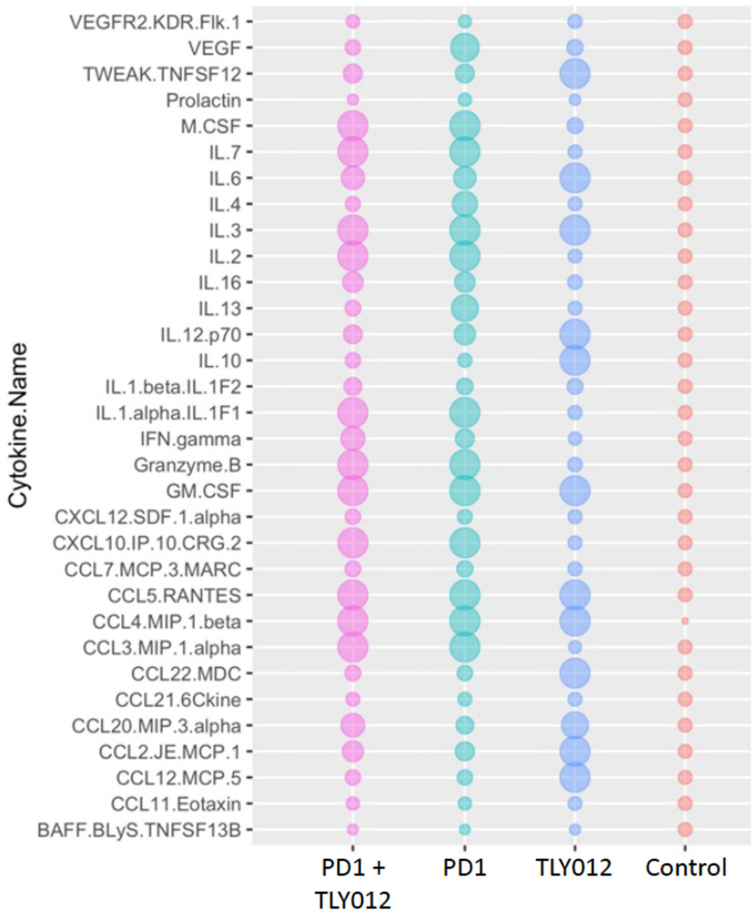


Figure 4. Long-term cytokine levels in KPC PDAC tumor-bearing C57Bl/6 mice for control, TLY012, anti-PD1, or dual therapy with TLY012 plus anti-PD1 as indicated. Three-month murine plasma cytokine levels are shown for the different treatment groups as indicated. Cytokine concentrations were normalized to the control group. Fold change is over control is represented by circle diameter.

Another limitation is that while we performed flow cytometric analysis of tumor tissue and spleens, as well as plasma cytokine profiling, we did not carry out immunohistochemical staining of tumors at early or late time points to provide further evidence of altered biomarkers and proposed mechanisms. We also did not test the requirement of NK- or T-cell alterations or cytokine alterations with regard to the observed anti-PDAC effects *in vivo*. We also did not evaluate the anti-fibrotic effects of TLY-012 as monotherapy or in combination with anti-PD1 *in vivo*.

It is noteworthy that IFNγ and CCL5 were increased in the combined treatment group (TLY012 + anti-PD1). However, how these changes may directly contribute to decreased tumor growth remains an open question for future research. The observed changes in T cells and NK cell populations mentioned above have not been tested as far as their requirement for tumor growth suppression. In addition future studies will need to unravel the importance of Tregs alterations such as their decrease in the spleen, but not in the tumor tissues.

Despite the limitations, our results provide a novel preclinical combination therapy of innate immune TLY012 and immune checkpoint blocker anti-mouse PD-1 that has potent efficacy in a KPC PDAC syngeneic C57Bl/6 mouse model and without evidence of toxicity. The results support the further exploration and clinical testing of TLY012 alone and in combination with anti-PD1 therapy in pancreatic cancer as well as other malignancies.

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Disclosure of conflict of interest

W.S.E-D. is the Scientific Founder of Oncoceutics, Inc., a subsidiary of Chimerix, and Founder of p53-Therapeutics, Inc., and SMURF-Therapeutics, Inc. Dr. El-Deiry has disclosed his relationships and potential conflict of interest to his academic institution/employer and is fully compliant with NIH and institutional policy that is managing this potential conflict of interest. The disclosed relationships by W.S.E-D.

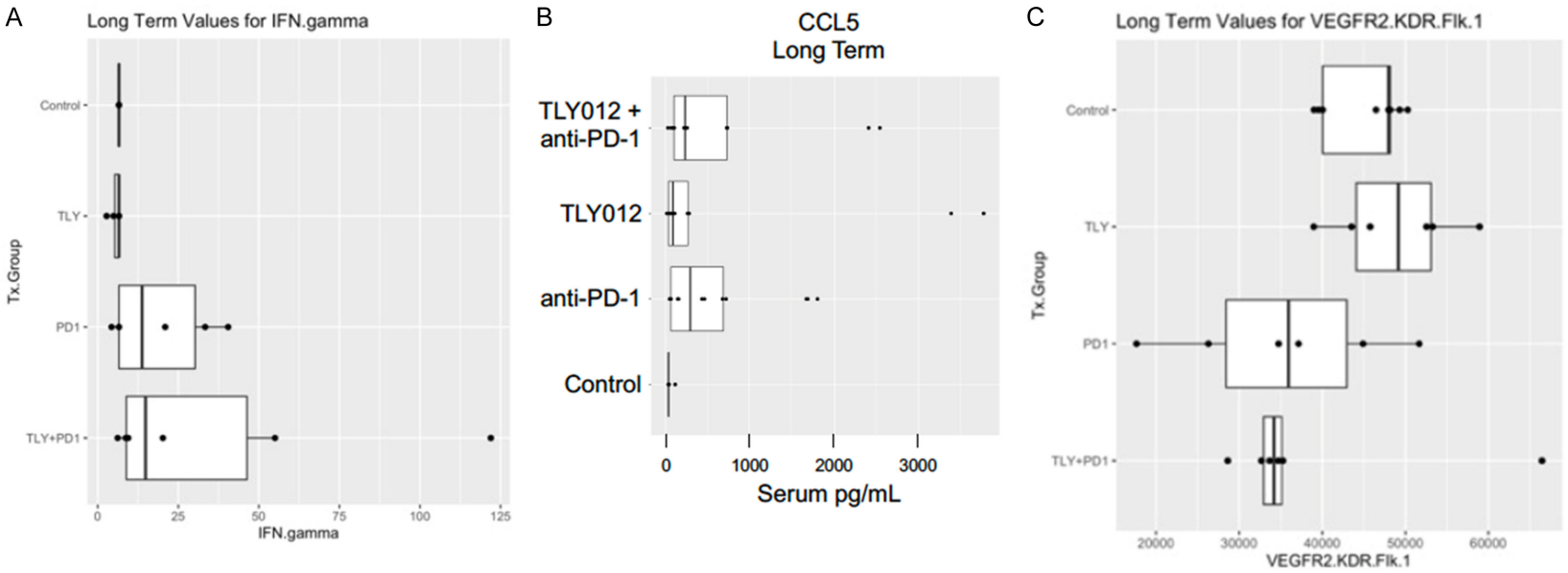

Figure 5. Alterations in selected cytokines at 3 months in KPC PDAC tumor-bearing C57Bl/6 mice for control, TLY012, anti-PD1, or dual therapy with TLY012 plus anti-PD1 as indicated. A. Plasma IFN-gamma levels at 3 months in pg/mL for control, TLY012, anti-PD1, or dual therapy with TLY012 plus anti-PD1 as indicated. B. CCL5 levels at 3 months in pg/mL for control, TLY012, anti-PD1, or dual therapy with TLY012 plus anti-PD1 as indicated. C. VEGFR2 levels at 3 months in pg/mL for control, TLY012, anti-PD1, or dual therapy with TLY012 plus anti-PD1 as indicated.

are not directly relevant to the present manuscript as none of the treatments used are owned or licensed by the entities he founded. S.L. is co-Founder, CEO and Chairman of D&D Pharmatech.

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