ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ESMO OPEN CANCER HORIZONS

ORIGINAL RESEARCH

Impact of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy on the outcomes in patients with refractory lung cancer

W. Yao[1], X. Zhao[2], Y. Gong[3], M. Zhang[3], L. Zhang[2], Q. Wu[2], L. Wu[2], Z. Fan[4], X. Yan[2*] & S. Jiao[1*]

[1]Medical School of Chinese PLA, Haidian, Beijing; [2]Department of Oncology, Chinese PLA General Hospital, Beijing; [3]Beijing DCTY® Biotech CO., LTD, Beijing; [4]Department of Oncology and Hematology, Shenzhen Third People's Hospital, School of Medicine, Southern University of Science and Technology, Shenzhen, Guangdong Province, China

Available online xxx

Background: PD-1/PD-L1 inhibitors in combination with chemotherapy are widely used in clinical practice. However, the ideal combined timing of them has not been fully explored.

Methods: In this study, simulation experiments to explore the impacts of the combination of anti-PD-1 antibody (anti-PD-1 Ab) on the cytotoxic effects of chemotherapeutic drugs in peripheral blood mononuclear cells were performed. In addition, the effects of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy on efficacy and safety were retrospectively analysed in patients with refractory lung cancer.

Results: Experiments *in vitro* showed that administering the anti-PD-1 Ab 3 days after chemotherapy (represented by dicycloplatin) resulted in significantly weaker cytotoxic effects on lymphocytes, compared with administering the anti-PD-1 Ab before or concurrent with chemotherapy. Moreover, data from 64 lung cancer patients treated with PD-1/PD-L1 inhibitors plus chemotherapy as a second- or higher-line therapy were retrospectively analysed. The results showed that administering PD-1/PD-L1 inhibitors 1-10 days (especially 3-5 days) after chemotherapy was associated with longer overall survival [17.3 months versus 12.7 months; hazard ratio (HR) = 0.58, 95% confidence interval (CI) 0.28-1.19, $P = 0.137$ in univariate analysis; HR = 0.36, 95% CI 0.16-0.80, $P = 0.012$ in multivariate analysis] and a trend of improved progression-free survival (5.1 months versus 4.2 months; HR = 0.81, 95% CI 0.42-1.54, $P = 0.512$) compared with administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy.

Conclusion: Our findings suggest that administering PD-1/PD-L1 inhibitors 1-10 days (especially 3-5 days) after chemotherapy is superior to administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy in patients with refractory lung cancer, but this result needs to be further explored by prospective studies.

Key words: immune checkpoint inhibitor, chemotherapy, combined timing, PBMC, lung cancer

INTRODUCTION

Immune checkpoint inhibitors (ICIs) have shown promising efficacy in the treatment of many types of cancers; however, only a small proportion of patients can benefit from ICI monotherapy.[1,2] Therefore, various combination strategies have been designed to enhance and broaden the clinical benefits of ICIs. Accumulating evidence indicates that chemotherapy can regulate antitumour immunity by inducing immunogenic cell death, modulating tumour-infiltrating lymphocytes, changing the expression of PD-L1 and so on, which provides a rationale for combining chemotherapy with immunotherapy to exert a synergistic effect.[3-7]

In recent years, numerous clinical studies have demonstrated that PD-1/PD-L1 inhibitors plus chemotherapy can exert a very good synergistic effect in patients with lung cancer, improving the objective response rate (ORR) and clinical outcomes.[8-14] These combination therapies are standard first-line treatment options for patients with lung cancer now and are widely used in clinical practice. However, the optimal sequencing and dosage of agents have not been fully explored.[5,6,15-19] Most, if not all, combination therapies are currently administered simultaneously and in standard doses, although the available evidence suggests that the timing and dosage of combined treatment may influence the eventual benefits of the treatment.[6,7,15,20]

*Correspondence to: Prof. Shunchang Jiao, Medical School of Chinese PLA, Haidian, Beijing; Department of Oncology, The Chinese PLA General Hospital, 28 Fuxing Road, Haidian, Beijing, 100853, China. Tel: +86-138-0138-0677; Fax: +86-010-6693-7592

E-mail: jiaosc301@163.com (S. Jiao).

*Dr Xiang Yan, Medical Oncology Department, The Chinese PLA General Hospital, 28 Fuxing Road, Haidian, Beijing, 100853, China. Tel: +86-135-8178-6750; Fax: +86-010-6693-7592

E-mail: yxiang301@sina.com (X. Yan).

For example, in a phase II trial of metastatic triple-negative breast cancer patients, upregulation of immune-related genes and increased T-cell infiltration in the tumour microenvironment were observed after low-dose induction of doxorubicin and cisplatin.[21] In two other phase II trials of carboplatin and paclitaxel combined with concurrent or phased ipilimumab, improved progression-free survival (PFS) and a trend towards an overall survival (OS) benefit were observed in the phased treatment group (carbo/taxol for two cycles followed by carbo/taxol/ipi for four cycles) but not in the concurrent group compared with chemotherapy alone in patients with small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC).[22,23] These studies suggest that administering chemotherapy up front to prime the immune system may be advantageous to maximize the efficacy of ICIs and may be superior to beginning chemotherapy and ICIs at the same time.

Another important aspect is that chemotherapeutic drugs are toxic to dividing cells, such as cancer cells and haematopoietic stem cells.[24,25] Meanwhile, lymphocytes also proliferate rapidly when activated, so they may also be damaged by chemotherapy. Moreover, we observed a significant increase in the cytotoxic effect of platinum in activated lymphocytes *in vitro* compared with unactivated lymphocytes. Given that PD-1/PD-L1 inhibitors can increase the proportion of activated lymphocytes in patients,[26,27] we speculated that chemotherapy, if used after or concurrent with PD-1/PD-L1 inhibitors, might abolish proliferating antitumour T cells activated by ICIs. By contrast, if chemotherapy was administered first and PD-1/PD-L1 inhibitors were given a few days later when the blood concentration of the chemotherapeutic drugs drops to a relatively low level, then the cytotoxic effect of chemotherapy on lymphocytes might be significantly weaker and the synergistic effect may be stronger. We suspect that there is a time point (or 'window' period) offering the best chance of combining PD-1/PD-L1 inhibitors and chemotherapy.

To test our hypothesis, we performed simulation experiments to explore the associations between the cytotoxicity of chemotherapeutic drugs and the activation states of peripheral blood mononuclear cells (PBMCs), and the effect of the combined timing of anti-PD-1 antibody (Ab) on the cytotoxicity of chemotherapy. Furthermore, 64 lung cancer patients treated with chemotherapy and PD-1/PD-L1 inhibitors as a second- or higher-line therapy were retrospectively analysed to verify our hypothesis and determine the best combined timing.

MATERIALS AND METHODS

In vitro study

Cells. Six sets of PBMCs were harvested from healthy donors and isolated by Ficoll-Paque Plus (GE Healthcare, Beijing, China) following the manufacturer's instructions. The Ethics Committee of PLA General Hospital approved the study, and written informed consent was provided by all donors.

Antibodies and reagents. The following antibodies were used for flow cytometric analyses: anti-CD45-KO, anti-CD3-FITC, anti-CD4-PC5, anti-CD8-APC, anti-PD-1-PE, and PD-1 isotype. The anti-PD-1-PE and PD-1 isotype antibodies were purchased from BD Pharmingen (San Diego, CA, USA). All the other antibodies were purchased from Beckman Coulter (Brea, CA, USA). The purified mouse anti-human CD3 monoclonal Ab (anti-CD3 Ab) was purchased from BD Pharmingen. Pembrolizumab was purchased from Merck & Co. (Shanghai, China) and was used as an anti-PD-1 monoclonal Ab (anti-PD-1 Ab) *in vitro*. Human recombinant PD-L1 proteins were purchased from Sino Biotechnology (BDA, Beijing, China). The CellTiter-Glo Luminescent Cell Viability Assay reagent and the Cytotoxicity LDH Assay Kit were purchased from Promega (Madison, WI, USA) and Dojindo (Shanghai, China), respectively. Dicycloplatin (DCP),[28] albumin-bound paclitaxel, and docetaxel were purchased from Beijing Xingda Pharmaceutical Research Cooperation (Beijing, China), CSPC (Shanghai, China), and Sanofi (Hangzhou, China), respectively. Cisplatin, pemetrexed, and etoposide were all purchased from Qilu Pharmaceutical (Jinan, China).

Cell culture and treatment. PBMCs were resuspended in RPMI-1640 medium (Gibco, Suzhou, China) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) and incubated at 37°C for 24 h before use in experiments. The culture plates were coated with the anti-CD3 Ab at 2 μg/ml overnight at 4°C. Precultured PBMCs were seeded at 1×10^5/ml in different types of culture plates according to the experimental needs and incubated at 37°C before being collected for flow cytometry analysis or other experiments.

Flow cytometry analysis. PBMCs stimulated by the anti-CD3 Ab were collected and incubated at room temperature with surface staining antibodies in phosphate-buffered saline for 30 min before being analysed on the BD FACSCanto II instrument. All data were analysed with FlowJo software (Tree Star, Inc.).[29]

Cell viability assay. Cell proliferation was examined using the CellTiter-Glo Luminescent Cell Viability Assay (Dojindo, Japan). In brief, pretreated PBMCs were seeded at 1×10^4 per well in 96-well culture plates coated with the anti-CD3 Ab, and chemotherapeutic drugs were added simultaneously at a full range of concentrations. After 72 h, CellTiter-Glo reagent was added to the culture medium, and the optical density was measured using a microplate luminescent detector (Centro XS^3 LB 960, Berthold Technologies, Bad Wildbad, Germany).

For the PD-L1 blocking assay, the recombinant PD-L1 protein was added at a range of concentrations along with PBMCs, and detection was performed 6 days later.[30]

For the anti-PD-1 Ab blocking assay, the recombinant PD-L1 protein and the anti-PD-1 Ab were added sequentially to final concentrations of 2 and 10 μg/ml, respectively, and PBMCs were cultured for up to 6 days and detected daily.

For the combination therapy assay, PBMCs were cultured in anti-CD3 Ab-coated 6-well plates and incubated with the recombinant PD-L1 protein at 2 μg/ml (and the anti-PD-1 monoclonal Ab at 10 μg/ml for the DCP + aPD-1 group) for 6 days in advance. Then, pretreated PBMCs were seeded at 1×10^4 per well in 96-well culture plates. DCP was added at a full range of concentrations, and the cells were cultured for 72 h before detection.

Cytotoxicity assay. Cytotoxicity was examined using the CellTiter-Glo Luminescent Cell Viability Assay and the Cytotoxicity LDH Assay kits. Three treatment groups were set according to the combined timing of anti-PD-1 Ab and chemotherapy: the immuno-advanced, concurrent, and immuno-delayed treatment groups. Each treatment group contained a combination (DCP + aPD-1) subgroup and a control (aPD-1) subgroup. PBMCs were seeded at 1×10^4 per well in 96-well culture plates coated with the anti-CD3 Ab, and the recombinant PD-L1 protein was added at a final concentration of 2 μg/ml simultaneously. The anti-PD-1 monoclonal Ab was added on day 0, day 3, and day 6 (2 hours before detection) to the immuno-advanced, concurrent, and immuno-delayed treatment groups, respectively, for a final concentration of 10 μg/ml. DCP was added on day 3 to the three combination subgroups for a final concentration of 10 μM. Finally, the cytotoxicity assay was performed using CellTiter-Glo reagent and the LDH Assay Kit following the manufacturer's instructions on day 6.

Retrospective study

Patients. Patients with metastatic lung cancer who received PD-1/PD-L1 inhibitors between June 2015 and June 2019 at Chinese People's Liberation Army General Hospital were screened. The inclusion criteria were as follows: (a) patients with histologically confirmed advanced lung cancer (adenocarcinoma, squamous cell carcinoma, or small-cell carcinoma) and (b) patients treated with PD-1/PD-L1 inhibitors plus chemotherapy as a second- or higher-line treatment. The exclusion criteria were as follows: (a) patients treated with any other drugs combined with chemotherapy and PD-1/PD-L1 inhibitors (such as anti-angiogenic drugs); (b) patients treated with chemotherapy and PD-1/PD-L1 inhibitors for less than two cycles; and (c) patients without an efficacy evaluation. This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital (S2018-203-01), and written informed consent was obtained from all patients.

Study objectives. The objectives of this retrospective analysis were to assess the impact of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy on patient OS and PFS, the ORR, and the safety profile. We collected the actual starting time of treatment with ICIs and chemotherapy from the physician's order sheets and determined the combined timing of ICIs and chemotherapy as the ICI starting time minus the chemotherapy starting time. OS was calculated as the time from the initiation of treatment to death from any cause or the last follow-up visit. PFS was calculated as the time from the initiation of treatment to disease progression, death from any cause, or the last follow-up visit. The clinical response was evaluated as complete response, partial response, stable disease, or progressive disease according to the RECIST criteria (version 1.1). Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0. All patients were followed up by telephone counselling and medical records review.

Statistical analysis

Numerical variables are expressed as the mean ± SD. The statistical significance of differences was evaluated by Student's *t*-test or ANOVA. Categorical variables are reported as frequencies and percentages and were compared using the chi-square test or Fisher's exact test. Receiver operating characteristic (ROC) curves were used to calculate the optimal cut-off value for the combined timing of PD-1/PD-L1 inhibitors and chemotherapy,[30] and the endpoint was based on OS in this study. Survival curves were estimated by Kaplan—Meier analysis, and comparisons between different groups were assessed by the log-rank test. Variables with $P \leq 0.2$ were included in the multivariate analysis. A Cox proportional hazards regression model was used to determine the independent prognostic factors. The data were analysed using SPSS statistical software (version 25.0; SPSS, IBM Corporation, USA) and GraphPad Prism (version 5.01; GraphPad Software, USA). All tests were two sided, and a P value ≤ 0.05 was considered statistically significant.

RESULTS

In vitro study

Kinetics of cell viability and surface PD-1 expression during PBMC activation. PD-1 is an inducible costimulatory molecule expressed on many kinds of immune cells (including T cells) upon activation.[15-17] As an initial step to understand the states of the anti-CD3 Ab-stimulated PBMCs we used in our experiments, the dynamic changes in cell viability and PD-1 expression during PBMC activation were analysed for 6 days. The results showed that the proliferation rate reached a peak on day 3 and was reduced in the next few days (Figure 1A and B). The dynamic changes in PD-1 expression on $CD3^+$ T cells were analysed by flow cytometry. The data showed that PD-1 expression on resting $CD3^+$ T cells was very low, but after stimulation, both the positive proportion and mean fluorescence intensity were upregulated and reached a peak on day 3 (on a par with the cell proliferation rate), followed by a reduction in the next few days (Figure 1C and D).

Association between the activation state and cytotoxic effect of DCP in PBMCs. The cytotoxic effect of DCP in PBMCs was examined by a luminescent cell viability assay. PBMCs stimulated with the anti-CD3 Ab for different lengths of time were used to represent lymphocytes in different activation states. As shown in Figure 1E and F, DCP inhibited PBMC proliferation in a concentration-dependent manner, and the 50% inhibitory concentration (IC_{50}) of DCP in PBMCs decreased as the stimulation time increased

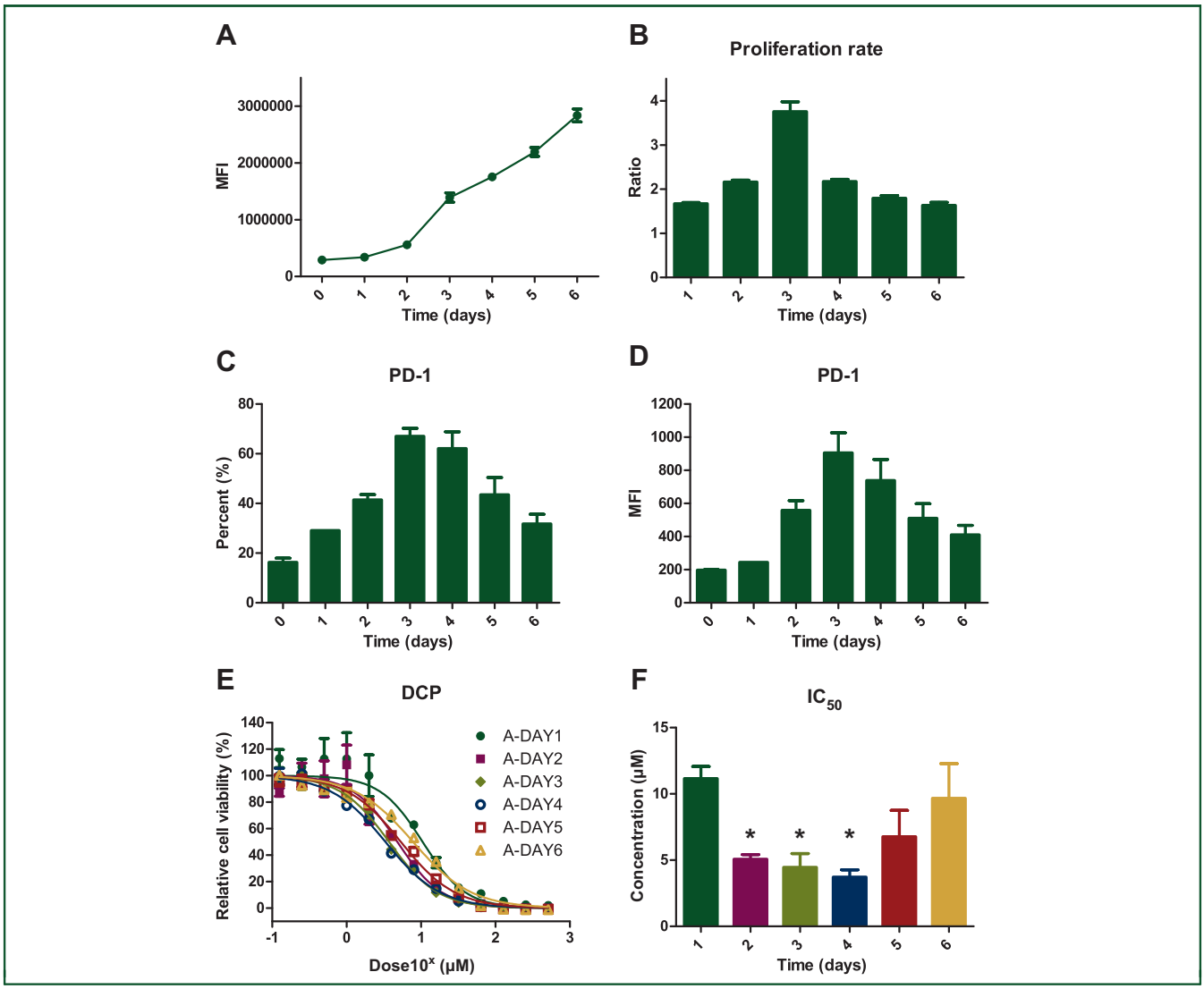

Figure 1. Association between the activation states and cytotoxic effects of dicycloplatin (DCP) in peripheral blood mononuclear cells (PBMCs) stimulated by the anti-CD3 antibody (anti-CD3 Ab).

(A, B) Proliferation of anti-CD3 Ab-stimulated PBMCs. Proliferation rate (day n) = MFI (day n)/MFI (day $n-1$). (C, D) PD-1 expression in anti-CD3 Ab-stimulated PBMCs. Dynamic changes in (C) the positive percent of PD-1 expression compared to the isotype control, and (D) the MFI of PD-1 expression on $CD3^+$ T cells after stimulation were detected by flow cytometry. (E, F) Cytotoxic effect of DCP in anti-CD3 Ab-stimulated PBMCs. PBMCs stimulated with the anti-CD3 Ab for different lengths of time were used to represent different activation states, and the cells were exposed to a full range of concentrations of DCP for 72 h before detection. (F) The 50% inhibitory concentration (IC_{50}) values of DCP in PBMCs stimulated for 2-6 days were compared with those in PBMCs stimulated for 1 day. The data are presented as mean ± SD of three independent experiments. *$P < 0.05$, statistically significant. MFI, mean fluorescence intensity.

and reached a minimum at days 3-4 followed by an increase in the next few days, indicating that the cytotoxic effect of DCP reached a peak at days 3-4, which is consistent with the trend observed in the PBMC proliferation rate and PD-1 expression. Therefore, the cytotoxic effect of DCP is positively correlated with the activation state in PBMCs.

Association between' the activation state and cytotoxic effect of multiple chemotherapy drugs in PBMCs. On the basis of the aforementioned findings, we chose day 0 and day 3 as our next experimental time points in the multiple chemotherapy drug studies. As shown in Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2021.100094, the IC_{50} values of DCP, cisplatin, pemetrexed, albumin-bound paclitaxel, docetaxel, and etoposide (drugs commonly used to treat lung cancer) in PBMCs activated for 3 days were all obviously lower than those in PBMCs activated for 0 days. Therefore, the cytotoxic effect of chemotherapy drugs commonly used in lung cancer is positively correlated with the activation state in PBMCs (i.e. chemotherapy drugs have a stronger cytotoxic effect on PBMCs in more active states).

Effect of the anti-PD-1 Ab on cell proliferation and the cytotoxic effect of DCP in PBMCs. The anti-PD-1 Ab showed no significant stimulatory effect on anti-CD3 Ab-stimulated PBMCs over a full range of concentrations (Figure 2A). The recombinant PD-L1 protein could significantly inhibit the

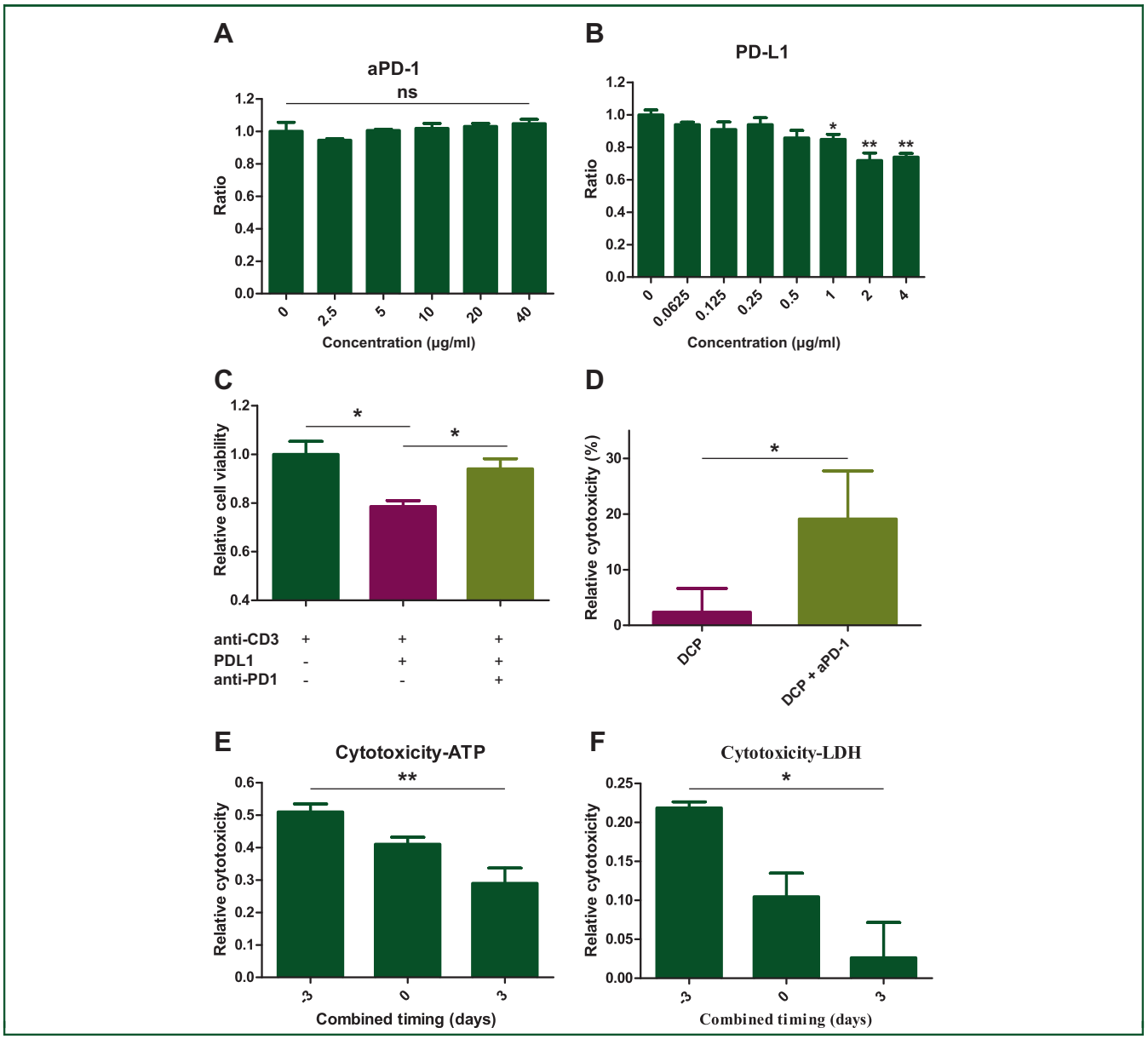

Figure 2. Impacts of the anti-PD-1 antibody (Ab) on cell proliferation and the cytotoxic effect of dicycloplatin (DCP) in peripheral blood mononuclear cells (PBMCs). (A) Effect of the anti-PD-1 Ab on anti-CD3 Ab-stimulated PBMCs. (B) Effect of the recombinant PD-L1 protein on anti-CD3 Ab-stimulated PBMCs. (C) Effect of the anti-PD-1 Ab on PD-L1-inhibited PBMCs. The recombinant PD-L1 protein and anti-PD-1 Ab were used at 2 μg/ml and 10 μg/ml, respectively, and cells were incubated for 6 days before detection. The graph shows a representative case from six sets of PBMCs, four of which demonstrated recovery from PD-L1-induced proliferation inhibition. (D) Effect of the anti-PD-1 Ab on the cytotoxicity of DCP in PD-L1-inhibited PBMCs. PBMCs pretreated with the PD-L1 protein (and anti-PD-1 Ab for the DCP + aPD-1 group) for 6 days were incubated with DCP for 72 h before detection. The graph shows the relative cytotoxicity of DCP at 1 μM. (E, F) Effect of the combined timing of anti-PD-1 Ab on the cytotoxicity of DCP in PD-L1-inhibited PBMCs. The cytotoxicity of DCP was measured by a (E) luminescent cell viability assay and (F) lactate dehydrogenase (LDH) assay. The relative cytotoxicity of DCP = [MFI (aPD-1) − MFI (DCP + aPD-1)]/MFI (aPD-1) for the cell viability assay. Each column indicates the mean ± SD of triplicate tests. ns, not statistically significant. *$P < 0.05$, **$P < 0.01$, statistically significant. ATP, adenosine triphosphate.

proliferation of anti-CD3 Ab-stimulated PBMCs when its concentration was >1 μg/ml (Figure 2B), so we chose 2 μg/ml as the next experimental concentration. As shown in Figure 3C and Supplementary Figure S2A, available at https://doi.org/10.1016/j.esmoop.2021.100094, the addition of 10 μg/ml anti-PD-1 Ab restored the proliferation inhibition caused by the PD-L1 protein, especially on day 6, and the difference was statistically significant in four of six sets of PBMCs. We next evaluated whether the promotion of proliferation caused by the anti-PD-1 Ab affects the cytotoxicity of chemotherapy drugs (represented by DCP) in PD-L1-inhibited PBMCs. Simulated experiments were performed, and the results showed that utilization of the anti-PD-1 Ab increased the cytotoxic effect of DCP in PD-L1-inhibited PBMCs, as we hypothesized (Figure 2D and Supplementary Figure S2B, available at https://doi.org/10.1016/j.esmoop.2021.100094).

Effect of the combined timing of anti-PD-1 Ab on the cytotoxic effect of DCP in PD-L1-inhibited PBMCs. Finally, a simulation experiment with different combined timing of anti-PD-1 Ab and chemotherapy (represented by DCP) was

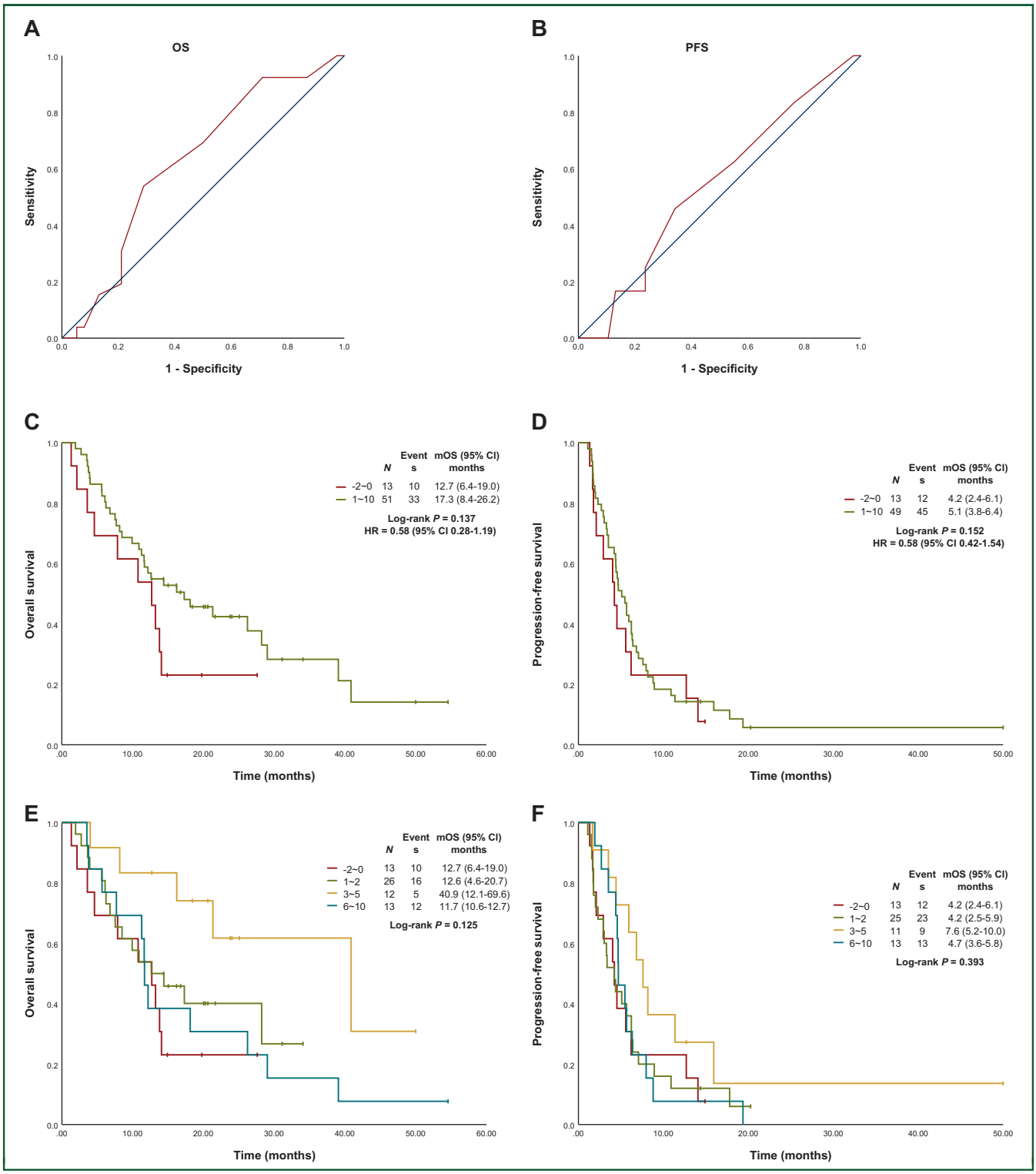

Figure 3. Survival analyses.
(A, B) Receiver operating characteristic (ROC) curve analyses of (A) overall survival and (B) progression-free survival for the optimal cut-off value of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy. (C-F) Patient survival. Kaplan—Meier survival curves comparing overall survival and progression-free survival between different combined-timing groups. CI, confidence interval; HR, hazard ratio; mOS, median overall survival; mPFS, median progression-free survival.

performed, and the schematic diagram of the treatment is shown in Supplementary Figure S2C, available at https://doi.org/10.1016/j.esmoop.2021.100094. The cytotoxic effect was measured by two different assays: the luminescent cell viability assay and lactate dehydrogenase assay. As shown in Figure 2E and F, the cytotoxic effect of DCP was reduced as the combined timing of anti-PD-1 Ab was delayed, and the immuno-delayed treatment group

Table 1. Analysis of clinical characteristics between patients with different combined timing of PD-1/PD-L1 inhibitors and chemotherapy

Characteristics	All patients	Combined timing (days)		*P* value
		−2 to 0	1 to 10	
Patients, *n*	64	13	51	
Age (years), *n* (%)				0.153
<60	31 (48.4)	4 (30.8)	27 (52.9)	
≥60	33 (51.6)	9 (69.2)	24 (47.1)	
Sex, *n* (%)				0.485
Male	49 (76.6)	9 (69.2)	40 (78.4)	
Female	15 (23.4)	4 (30.8)	11 (21.6)	
Smoking status, *n* (%)				0.203
Never	22 (34.4)	7 (53.8)	15 (29.4)	
Former	33 (51.6)	4 (30.8)	29 (56.9)	
Current	9 (14.1)	2 (15.4)	7 (13.7)	
Performance status (KPS), *n* (%)				0.528
<90	11 (17.2)	3 (23.1)	8 (15.7)	
≥90	53 (82.8)	10 (76.9)	43 (84.3)	
TNM stage, *n* (%)				0.557
III	8 (12.5)	1 (7.7)	7 (13.7)	
IV	56 (87.5)	12 (92.3)	44 (86.2)	
Brain metastasis, *n* (%)				0.878
No	55 (85.9)	11 (84.6)	44 (86.2)	
Yes	9 (14.1)	2 (15.4)	7 (13.7)	
Histology, *n* (%)				0.087
Adenocarcinoma	27 (42.2)	9 (69.2)	18 (35.3)	
Squamous cell carcinoma	18 (28.1)	2 (15.4)	16 (31.3)	
Small-cell carcinoma	19 (29.7)	2 (15.4)	17 (33.3)	
EGFR mutation status, *n* (%)				0.189
Negative	27 (42.2)	8 (61.5)	19 (37.2)	
Positive	8 (12.5)	2 (15.4)	6 (11.8)	
Unknown	29 (45.3)	3 (23.1)	26 (51.0)	
ALK rearrangement status, *n* (%)				0.182
Negative	29 (45.3)	8 (61.5)	21 (41.2)	
Positive	2 (3.1)	1 (7.7)	1 (2.0)	
Unknown	33 (51.6)	4 (30.8)	29 (56.9)	
PD-L1 TPS, *n* (%)				0.105
<1%	4 (6.2)	0 (0.0)	4 (7.8)	
1%-49%	9 (14.1)	3 (23.1)	6 (11.8)	
≥50%	9 (14.1)	4 (30.8)	5 (9.8)	
Unknown	42 (65.6)	6 (46.2)	36 (70.6)	
Line of therapy, *n* (%)				0.953
Second	30 (46.9)	6 (46.2)	24 (47.1)	
Third or higher	34 (53.1)	7 (53.8)	27 (52.9)	
Type of chemotherapy, *n* (%)				0.291
Single-agent chemotherapy	36 (56.3)	9 (69.2)	27 (52.9)	
Platinum-based doublet chemotherapy	28 (43.8)	4 (30.8)	24 (47.1)	
Immune checkpoint inhibitors, *n* (%)				0.817
Pembrolizumab	33 (51.6)	8 (61.5)	25 (49.0)	
Nivolumab	22 (34.4)	4 (30.8)	18 (35.3)	
Other anti-PD-1 agents	8 (12.5)	1 (7.7)	7 (13.7)	
Atezolizumab	1 (1.6)	0 (0.0)	1 (2.0)	

PD-L1 TPS, PD-L1 tumour proportion score.

(combined timing: 3 days after chemotherapy) had the weakest cytotoxic effect on PD-L1-inhibited PBMCs. Moreover, the results from the two different assays were consistent.

Retrospective study

Patient characteristics. The baseline patient characteristics are summarized in Table 1. A total of 64 patients were included in the study. The median age was 60 (range 38-86 years) years; 49 (76.6%) patients were male, and 15 (23.4%) were female. Among these patients, 27 (42.2%) had adenocarcinoma, 18 (28.1%) had squamous cell carcinoma, and 19 (29.7%) had small-cell carcinoma. All patients were treated with PD-1/PD-L1 inhibitors plus chemotherapy, and the most commonly used chemotherapy regimen was taxane (especially albumin-bound paclitaxel). Before the combination therapy, all 64 patients had received one or more lines of therapy: 30 (46.9%) had received one frontline therapy and 34 (53.1%) had received two or more lines of therapy. Follow-up ended on 20 September 2020, with a median follow-up time of 13.5 (range 1.3-54.6) months.

Selection of the optimal cut-off value for the combined timing of PD-1/PD-L1 inhibitors and chemotherapy. We attempted to establish the optimal cut-off value for the combined timing of PD-1/PD-L1 inhibitors and chemotherapy through ROC curve analysis. As shown in Figure 3A and B, the areas under the curves for OS and PFS were 0.626 ($P = 0.089$) and 0.544 ($P = 0.563$), respectively. The optimal cut-off value for the prediction of OS was 0 days, and thus, the patients were divided into two groups: 13 (20.3%) patients treated with ICIs $\leq$ 0 days (−2 to 0 days) after chemotherapy and 51 (79.7%) patients treated with ICIs $>$ 0 days (1-10 days) after chemotherapy. To explore the optimal combined timing, we further classified the patients into four treatment subgroups, −2 to 0 days, 1-2 days, 3-5 days, and 6-10 days, according to the trend of the ROC curve.

Association between survival and the combined timing of PD-1/PD-L1 inhibitors and chemotherapy. The clinical characteristics were comparable between the two treatment groups (Table 1). The correlation between the combined timing and survival is shown in Figure 3. A trend of longer OS was observed in patients from the 1-10-day treatment group versus those from the −2- to 0-day treatment group [median 17.3 months versus 12.7 months; hazard ratio (HR) = 0.58, 95% confidence interval (95% CI) 0.28-1.19; $P = 0.137$; Figure 3C]. The median PFS rates of patients in the 1-10-day and −2- to 0-day treatment groups were 5.1 and 4.2 months, respectively, and the difference was not statistically significant [HR = 0.81, 95% CI 0.42-1.54; $P = 0.512$; Figure 3D].

Further analysis of the four treatment subgroups showed that patients treated with PD-1/PD-L1 inhibitors 3-5 days after chemotherapy had a significantly prolonged OS compared with patients treated with PD-1/PD-L1 inhibitors −2 to 0 days after chemotherapy [median 40.9 months versus 12.7 months; HR = 0.27, 95% CI 0.09-0.82; $P = 0.021$; Figure 3E]. There was also a trend of longer PFS rates in patients from the 3-5-day group than in those from the −2- to 0-day group (median 7.6 months versus 4.2 months; HR = 0.51, 95% CI 0.21-1.22; $P = 0.131$; Figure 3F).

Table 2. Univariate and multivariate analyses of overall survival

Parameter	Univariate analysis			Multivariate analysis					
				Model 1			Model 2		
	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Performance status									
90 versus <90	2.800	0.996-7.870	0.051	3.677	1.277-10.591	**0.016**	2.870	0.94-8.768	0.064
Brain metastasis									
Yes versus no	2.358	1.078-5.156	0.032	2.149	0.873-5.288	0.096	1.962	0.785-4.905	0.149
Histology									
SCLC versus NSCLC	1.664	0.875-3.165	0.121	2.088	0.951-4.586	0.067	2.042	0.922-4.521	0.078
Combined timing-1									
1-10 versus −2 to 0 days	0.578	0.280-1.191	0.137	0.360	0.162-0.800	**0.012**			
Combined timing-2			0.125						0.062
1-2 versus −2 to 0 days	0.663	0.299-1.469	0.311				0.398	0.166-0.957	**0.040**
3-5 versus −2 to 0 days	0.272	0.090-0.818	0.021				0.230	0.072-0.731	**0.013**
6-10 versus −2 to 0 days	0.781	0.326-1.870	0.579				0.454	0.174-1.182	0.106

HR, hazard ratio; 95% CI, 95% confidence interval; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.
The bold values, which were statistically significant in multivariate analysis.

From the univariate Cox regression analyses, Karnofsky performance status (KPS; $P = 0.051$), brain metastasis ($P = 0.032$), histology ($P = 0.121$), and combined timing ($P = 0.137$) were more correlated with OS ($P < 0.2$, Table 2) and were further investigated in multivariate analyses. In the multivariate analyses, KPS ($P = 0.016$) and combined timing of PD-1/PD-L1 inhibitors and chemotherapy ($P = 0.012$) were both independent indictors for OS in model 1. In addition, in multivariable model 2, combined timing of 3-5 days (HR = 0.23, 95% CI 0.07-0.73; $P = 0.013$) and 1-2 days (HR = 0.40, 95% CI 0.17-0.96; $P = 0.04$) was also an independent indictor for superior OS compared with the −2- to 0-day treatment group (Table 2). Moreover, the trend of the OS benefit in the 1-10-day treatment group was observed in all the subgroup analyses, although most of the differences were not statistically significant (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2021.100094).

Tumour response and adverse events. There was no significant difference between the two combined-timing groups in ORR (35.3% versus 38.5%, $P = 0.832$) or disease control rate (DCR, 86.2% versus 84.6%, $P = 0.878$). However, the ORR of the 3-5-day treatment subgroup was higher than that of the other combined-timing subgroups, as shown in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2021.100094 (50.0% versus 38.5%, 34.6% and 23.1%). Immune-related adverse events of grade 3-4 occurred in 0 of 13 patients (0.0%) who received PD-1/PD-L1 inhibitors −2 to 0 days after chemotherapy and in 4 of 51 patients (7.8%) who received PD-1/PD-L1 inhibitors 1-10 days after chemotherapy ($P = 0.297$). The granulocyte colony-stimulating factor utilization rates of the −2- to 0-day and 1-10-day groups were comparable (38.5% and 33.3%, $P = 0.728$).

DISCUSSION

This study represents the first exploration of the best combined timing of PD-1/PD-L1 inhibitors and chemotherapy in patients with refractory lung cancer. In simulation experiments *in vitro*, we observed that the cytotoxic effects of chemotherapeutic drugs (commonly used in lung cancer) were all positively correlated with the activation state in PBMCs. Thus the promotion of proliferation caused by the anti-PD-1 Ab increased the cytotoxic effect of chemotherapeutic drugs (represented by DCP) in PD-L1-inhibited PBMCs, as we had surmised. Subsequently, combined-timing experiments were performed, which showed that administering the anti-PD-1 Ab 3 days after DCP resulted in significantly less damage to lymphocytes, compared with administering the anti-PD-1 Ab 3 days before or concurrent with chemotherapy, indicating that administering the anti-PD-1 Ab a few days after chemotherapy may exert a better synergistic effect in patients.

In a relevant *in vivo* study, the antitumour effect of cyclophosphamide combined with a CTLA-4 inhibitor was examined in murine tumour models with immunogenic CT26 colon carcinoma. Drastic tumour regression was observed when the anti-CTLA-4 Ab was injected 1-3 days after cyclophosphamide (100 mg/kg). However, when the injection order was reversed, the antitumour effect diminished, and apoptosis of tumour-specific $CD8^+$ T cells increased.[31] The observations from this study support the results we obtained *in vitro*, that is, administering ICIs a few days after chemotherapy could cause less damage to lymphocytes (especially T cells activated by ICIs) and might result in a better synergistic effect than administering ICIs before or concurrent with chemotherapy.

To further validate the results obtained *in vitro*, we retrospectively analysed the associations between the combined timing of PD-1/PD-L1 inhibitors and chemotherapy and the treatment efficacy and safety in patients with refractory lung cancer. The results showed that administering PD-1/PD-L1 inhibitors 1-10 days (especially 3-5 days) after chemotherapy was associated with significantly improved OS (17.3 months versus 12.7 months; HR = 0.58, 95% CI 0.28-1.19, $P = 0.137$ in univariate analysis; HR = 0.36, 95% CI 0.16-0.80, $P = 0.012$ in multivariate analysis) and a trend of longer PFS (5.1 months versus 4.2 months; HR = 0.81, 95% CI

0.42-1.54, $P = 0.512$) compared with administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy as a second- or higher-line therapy, although there was no significant difference in ORR between the two groups (35.3% versus 38.5%, $P = 0.832$). The observations from the retrospective study were consistent with the results of the *in vitro* experiments that administering PD-1/PD-L1 inhibitors after chemotherapy was superior to administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy, which indicated the importance of the combined timing in combination therapy and the necessity to further explore this topic.

There have been many clinical trials of chemotherapy in combination with ICIs in lung cancer, which have demonstrated a median OS of 15.9-18.6 months and a median PFS of 6.4-7.0 months, as a first-line therapy in NSCLC.[9,14] In addition, a median OS of 12.3 months and a median PFS of 5.2 months have been reported in extensive SCLC from the IMpower133 trial.[10] In our study, chemotherapy plus ICIs reached a median OS of 13.8 months (95% CI 9.5-18.1 months), a median PFS of 4.7 months (95% CI 3.5-5.8 months), and an ORR of 35.9% in all patients. The median OS and PFS in our study were shorter than those in the aforementioned clinical trials, mainly because the combination treatment was performed as a second- or higher-line therapy in our study, rather than as a first-line therapy. In addition, 56.3% of the total population (36 out of 64) was treated with single-agent chemotherapy in the combination therapy rather than platinum-based doublet chemotherapy, which also contributed to the inferior survival.

There are some limitations in this study. First, in the basic research, we only performed *in vitro* experiments to simulate the treatment of chemotherapy combined with PD-1/PD-L1 inhibitors and its effect on lymphocytes and did not have a chance to perform *in vivo* experiments to verify the findings. The good thing is there was a relevant *in vivo* study which confirmed our findings.[31] Second, the clinical study was retrospective, and the sample size was small because of the limited application time of the combination therapy; thus some bias and confounding factors were inevitable. The association between the combined timing of treatment and OS did not reach statistical significance ($P = 0.137$) in the univariate analyses, likely due to the small sample size. Third, we included both NSCLC and SCLC patients in our study to expand the sample size, although histology type is an important factor affecting survival. Nevertheless, we performed multivariate and subgroup analyses to exclude the influence of histology on survival, and the trends in the histology subgroups were all consistent with the results observed in all patients. However, whether the combined timing of PD-1/PD-L1 inhibitors and chemotherapy has a similar association with OS in first-line treatment needs further investigation.

In conclusion, we observed that administering PD-1/PD-L1 inhibitors 1-10 days (especially 3-5 days) after chemotherapy was superior to administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy as a second- or higher-line therapy in refractory lung cancer patients. These findings need to be further explored by prospective studies.

FUNDING

This work was supported by the National Natural Science Foundation of China, China (grant numbers 81972734, 81972681).

DISCLOSURE

The authors have declared no conflicts of interest.

REFERENCES

1. Gettinger S, Horn L, Jackman D, et al. Five-year follow-up of nivolumab in previously treated advanced non-small-cell lung cancer: results from the CA209-003 study. *J Clin Oncol* 2018;36(17):1675-84.
2. Shergold AL, Millar R, Nibbs RJB. Understanding and overcoming the resistance of cancer to PD-1/PD-L1 blockade. *Pharmacol Res* 2019;145:104258.
3. Hato SV, Khong A, de Vries IJM, Lesterhuis WJ. Molecular pathways: the immunogenic effects of platinum-based chemotherapeutics. *Clin Cancer Res* 2014;20(11):2831-7.
4. Liu WM, Fowler DW, Smith P, Dalgleish AG. Pre-treatment with chemotherapy can enhance the antigenicity and immunogenicity of tumours by promoting adaptive immune responses. *Br J Cancer* 2010;102(1):115-23.
5. Pusuluri A, Wu D, Mitragotri S. Immunological consequences of chemotherapy: single drugs, combination therapies and nanoparticle-based treatments. *J Control Release* 2019;305:130-54.
6. Pfirschke C, Engblom C, Rickelt S. Immunogenic chemotherapy sensitizes tumors to checkpoint blockade therapy. *Immunity* 2016;44(2):343-54.
7. Wu X, Feng Q-M, Wang Y, Shi J, Ge H-L, Di W. The immunologic aspects in advanced ovarian cancer patients treated with paclitaxel and carboplatin chemotherapy. *Cancer Immunol Immunother* 2010;59(2):279-91.
8. Paz-Ares L, Dvorkin M, Chen Y, et al. Durvalumab plus platinum—etoposide versus platinum—etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. *Lancet* 2019;394(10212):1929-39.
9. Paz-Ares L, Luft A, Vicente D, et al. Pembrolizumab plus chemotherapy for squamous non-small-cell lung cancer. *N Engl J Med* 2018;379(21):2040-51.
10. Horn L, Mansfield AS, Szczęsna A, et al. First-Line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. *N Engl J Med* 2018;379(23):2220-9.
11. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med* 2018;379(22):2108-21.
12. Langer CJ, Gadgeel SM, Borghaei H, et al. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. *Lancet Oncol* 2016;17(11):1497-508.
13. Gandhi L, Rodríguez-Abreu D, Gadgeel S, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N Engl J Med* 2018;378(22):2078-92.
14. West H, McCleod M, Hussein M, et al. Atezolizumab in combination with carboplatin plus nab-paclitaxel chemotherapy compared with chemotherapy alone as first-line treatment for metastatic non-squamous non-small-cell lung cancer (IMpower130): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol* 2019;20(7):924-37.
15. Brown JS, Sundar R, Lopez J. Combining DNA damaging therapeutics with immunotherapy: more haste, less speed. *Br J Cancer* 2017;118(3):312-24.

16. Nisticò P, Capone I, Palermo B, et al. Chemotherapy enhances vaccine-induced antitumor immunity in melanoma patients. *Int J Cancer* 2009;124(1):130-9.
17. Emens LA, Middleton G. The interplay of immunotherapy and chemotherapy: harnessing potential synergies. *Cancer Immunol Res* 2015;3(5):436-43.
18. Chen G, Emens LA. Chemoimmunotherapy: reengineering tumor immunity. *Cancer Immunol Immunother* 2013;62(2):203-16.
19. Charpidou A, Vathiotis I, Gkiozos I, Syrigos K. Exceptional antitumor responses beyond immune checkpoint inhibition in non-small cell lung cancer patients: insights into optimal therapy sequencing. *J Thorac Dis* 2019;11(3):E25-31.
20. Arrieta O, Barrón F, Ramírez-Tirado LA, et al. Efficacy and safety of pembrolizumab plus docetaxel vs docetaxel alone in patients with previously treated advanced non-small cell lung cancer: the PROLUNG phase 2 randomized clinical trial. *JAMA Oncol* 2020;6(6):856-64.
21. Voorwerk L, Slagter M, Horlings HM, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med* 2019;25(6):920-8.
22. Reck M, Bondarenko I, Luft A, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer: results from a randomized, double-blind, multicenter phase 2 trial. *Ann Oncol* 2013;24(1):75-83.
23. Lynch TJ, Bondarenko I, Luft A, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. *J Clin Oncol* 2012;30(17):2046-54.
24. Crawford J, Dale DC, Lyman GH. Chemotherapy-induced neutropenia: risks, consequences, and new directions for its management. *Cancer* 2004;100(2):228-37.
25. Barreto JN, McCullough KB, Ice LL, Smith JA. Antineoplastic agents and the associated myelosuppressive effects: a review. *J Pharm Pract* 2014;27(5):440-6.
26. Kamphorst AO, Pillai RN, Yang S, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci U S A* 2017;114(19):4993-8.
27. Kamphorst AO, Wieland A, Nasti T, et al. Rescue of exhausted CD8 T cells by PD-1—targeted therapies is CD28-dependent. *Science* 2017;355:1423-7.
28. Li GQ, Chen X-G, Wu X-P, et al. Effect of dicycloplatin, a novel platinum chemotherapeutical drug, on inhibiting cell growth and inducing cell apoptosis. *PLoS One* 2012;7(11):e48994.
29. Xing K, Gu B, Zhang P, Wu X. Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC Immunol* 2015;16:39.
30. Tong YS, Tan J, Zhou X-L, Song Y-Q, Song Y-J. Systemic immune-inflammation index predicting chemoradiation resistance and poor outcome in patients with stage III non-small cell lung cancer. *J Transl Med* 2017;15(1):221.
31. Iida Y, Harashima N, Motoshima T, Komohara Y, Eto M, Harada M. Contrasting effects of cyclophosphamide on anti-CTL-associated protein 4 blockade therapy in two mouse tumor models. *Cancer Sci* 2017;108(10):1974-84.