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Original Article

Synergistic effect of programmed death-1 inhibitor and programmed death-1 ligand-1 inhibitor combined with chemotherapeutic drugs on DLBCL cell lines *in vitro* and *in vivo*

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL). Chemotherapy is one of the main treatments for cancer, but the antitumor effect of chemotherapeutic drugs is affected by the patient's immune status. The programmed cell death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) axis is an important central checkpoint in tumor progression. The present study demonstrated a significant synergistic effect of PD-1 inhibitor and oxaliplatin, cisplatin, etoposide, cytarabine, ifosfamide and carboplatin. There was no difference in cytotoxicity between the groups with or without PD-L1 inhibitor. It was also observed that cytotoxicity of T cells combined with PD-1 inhibitor against DLBCL cells was inhibited by dexamethasone addition to the culture system at 24, 48 and 72 h. There was no difference in cytotoxicity between the group of dexamethasone added at 96 h and the group without dexamethasone at 96 h. Then, we selected a PD-1 inhibitor combined with a chemotherapeutic regimen in a Pfeiffer cell mouse xenograft model. At 21 days, the reduction in tumor size was more obvious in the DHAP combined with PD-1 inhibitor group (dexamethasone after 96 h of PD-1) compared with that in the DHAP (P=0.007), the PD-1 inhibitor (P=0.001) and the DHAP combined with PD-1 inhibitor (dexamethasone after 24 h of PD-1) (P=0.005) groups. However, the reduction in tumor size was more obvious in the GemOx combined with PD-1 inhibitor group compared with that in the GemOx group (P=0.037). Therefore, the present study demonstrated the synergistic effects of PD-1 inhibitor combined with chemotherapeutic regimens in DLBCL.

Keywords: PD-1 inhibitor, PD-L1 inhibitor, T cells, Pfeiffer cells, chemotherapy

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL). Rituximab (CD20 monoclonal antibody, mAb) plus cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) have significantly increased the overall survival (OS) and the 5-year progression-free survival (PFS) rates [1, 2]. However, several patients with NHL, particularly those with DLBCL, develop relapsed/refractory (R/R) disease after standard combined chemotherapy. Therefore, a novel therapeutic strategy is needed to improve the curative effect and prolong patient survival. The programmed cell death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) or PD-L2 axis is an important central checkpoint in tumor progression [3]. PD-1 is an immune checkpoint that may prevent the immune system from killing cancer cells [4]. Previous research revealed that the PD-1 gene is overexpressed in patients with DLBCL [5]. The expression of the PD-1 gene increases with tumor aggressiveness, and is an independent factor associated with OS. In recent years, it was reported that there is a correlation between the PD-1/PD-L1 axis and the prognosis of DLBCL. High PD-L1 cell numbers were found to be associated with poor event-free survival [6]. Therefore, PD-1 inhibitors may be considered as an immunological target for the treatment of R/R DLBCL.

Other studies have demonstrated that the OS and PFS of patients with advanced non-small cell lung cancer (NSCLC) treated with PD-1 in-

hibitors combined with gemcitabine and cisplatin were longer compared with those following treatment with gemcitabine or cisplatin alone [7, 8]. In the present study, the combined effects of PD-1 inhibitors and cisplatin, carboplatin, oxaliplatin, gemcitabine, isocyclophosphamide, mitoanthraquinone, etoposide, cytarabine and dexamethasone were investigated. Furthermore, the combined effect of PD-1 inhibitors and chemotherapeutic drugs were observed in vitro and in vivo, in the hope of identifying the chemotherapeutic drugs that are more effective in combination with PD-1 inhibitors.

Materials and methods

Primary cells, cell lines, mice, PD-1 inhibitor and PD-L1 inhibitor

$CD3^+$ T cells in this experiment were isolated from five healthy donors. Ethics approval was granted by the IRB of the hospital and informed consent was obtained from the donors. DLBCL cell lines including Pfeiffer, U2932, OCI-ly7 and OCI-ly cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI-1640 medium containing 15% fetal bovine serum, 1% penicillin/streptomycin, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate. Female CAnN.Cg-Foxn1nu/CrlVR mice, aged 6-8 weeks and weighing 20±1.8 g (Beijing Vitonlihua Experimental Animal Technology Co., Ltd, Beijing, China), were selected for the *in vivo* study. All animal experiments were approved by the Ethics Committee of Tianjin First Central Hospital. The PD-1 inhibitor was OPDIVO (nivolumab) and the PD-L1 inhibitor was purchased from MCE (MedChemExpress).

Isolation of PBMCs and T-cell culture in vitro

PBMCs from five healthy donors were isolated from the buffy coat (New York Blood Center, New York, NY, USA) by Ficoll density gradient centrifugation (500 × g for 10 min at room temperature). The $CD3^+$ T cells were selected by MACS using CD3 microbeads (Miltenyi Biotec, Inc., Cambridge, MA, USA) from the PBMCs. Then, $CD3^+$ T cells were cultured in T-cell medium X-Vivo 15 (Lonza Group, Ltd., Basel, Switzerland) supplemented with 250 IU/ml interleukin-2 (IL-2; Proleukin®; Novartis International AG, Basel, Switzerland) every 2 days at 37°C in a humidified incubator with 4% CO_2. The T cells were harvested on day 12 after isolation and culture *in vitro*.

Selection of the proportion of T cells and DLBCL cell lines in mixed culture

T cells were mixed with DLBCL cell lines at an effector:target ratio (E:T) of 1:1, 2:1, 5:1 and 10:1. The cytotoxic activity was detected using lactate dehydrogenase (LDH) cytotoxicity Assay Kit (Dojindo Molecular Technologies, Inc.) at 490 nm to select the proportion of T cells to Pfeiffer cell lines in the following experiment.

Concentration selection of PD-1 or PD-L1 inhibitor

Different doses of PD-1 inhibitor (18, 36, 72 and 144 μg/ml) were added in the co-culture system of T cells and DLBCL cell lines [9, 10]. Different doses of PD-L1 inhibitor (10, 50, 100 and 200 μg/ml) were added in the co-culture system of T cells and DLBCL cell lines. The cytotoxic activity was detected by the LDH method to select the concentration of PD-1 or PD-L1 inhibitor in the following experiments.

Selection of the concentration of chemotherapeutic drugs

The dose of each chemotherapeutic drug in the in vitro experiment was determined based on the clinical suggested doses and published literature. The concentrations of chemotherapy drugs selected for the present study were as follows: Cisplatin at 0.3 mg/ml, cytarabine at 0.3 mg/ml, gemcitabine at 0.6 mg/ml, carboplatin at 0.3 mg/ml, oxaliplatin at 0.04 mg/ml, etoposide at 0.3 mg/ml, isocyclophosphamide at 3 mg/ml and mitoxanthraquinone at 6 μg/ml. We set different concentration gradients of the chemotherapy drug to observe the cytotoxic activity against DLBCL cell lines.

Flow cytometry

The expression of CD3 in T cells, PD-1 in $CD3^+$ T cells and PD-L1 in DLBCL cell lines was analyzed using flow cytometry. The expression of CD3 in T cells was analyzed using anti-CD3-APC (Beckman Coulter, Inc.). The expression of PD-1 in $CD3^+$ T cells was analyzed using anti-CD279-FITC (Miltenyi Biotec, Inc.). The expression of CD19 and PD-L1 in Pfeiffer cells were ana-

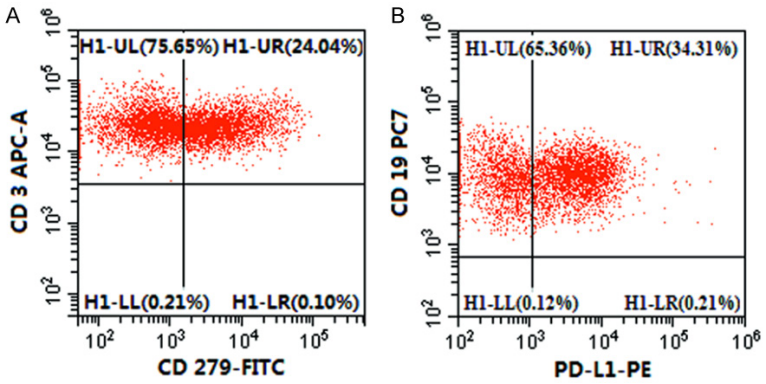


Figure 1. Expression of PD-1 on CD3$^+$ T cells and PD-L1 on DLBCL cells. A. Expression of PD-1 on CD3$^+$ T cells of the five healthy donors. B. Expression of PD-L1 on Pfeiffer cells.

lyzed using anti-CD19 PC7 and anti-PD-L1-PE (Beckman Coulter, Inc.).

Cytotoxicity against DLBCL cell lines in vitro

The T cells were pretreated with PD-1 inhibitor in the absence of supplemented cytokines for 24 h. The DLBCL cell lines were pretreated with PD-L1 inhibitor for 24 h. There were four groups in the present study: i) T cells + DLBCL cell lines (E:T=1:1), ii) T cells + DLBCL cell lines + chemotherapeutic drug, iii) T cells + DLBCL cell lines + PD-1 inhibitor (or PD-L1 inhibitor) and iv) T cells + DLBCL cell lines + PD-1 inhibitor (or PD-L1 inhibitor) + chemotherapeutic drug. The co-culture time of each group was 24, 48 and 72 h. Cytotoxicity was detected using the LDH method at 490 nm at 0, 24 and 48 h. The experiment *in vitro* was repeated three times.

Xenograft tumor model

Female 6-8-week-old CAnN.Cg-Foxn1nu/CrlVR (BALB/c) mice, weighing 20.25±1.51 g (n=24, Beijing Vitonlihua Experimental Animal Technology Co., Ltd, Beijing, China), were injected with 1 × 10^7 Pfeiffer cells transduced with luciferase (Shanghai Suer Biotechnology Co.) by subcutaneous injection. The mice were monitored for established tumors by bioluminescence imaging (BLI) *in vivo*. Upon confirmation of engraftment after 25 days, the mice were randomized and treated by tail vein injection as the following groups: i) Control group (no treatment), ii) DHAP/GemOx group, iii) PD-1 inhibitor group and iv) DHAP/GemOx combined with PD-1 inhibitor group. The DHAP combined with PD-1 inhibitor group was divided into two subgroups: the dexamethasone after 24 or 96 h of PD-1 groups. The DHAP chemotherapy regimen includes cisplatin, cytarabine and dexamethasone. The GemOx chemotherapy regimen includes gemcitabine and oxaliplatin. The dose of the DHAP/GemOx chemotherapy regimen was half of the clinically recommended dose according to the NCCN 2020 guidelines. The mice of the PD-1 inhibitor group received 5 × 10^6/kg of T cells and 3 mg/kg of PD-1 inhibitor on the first day of the combination therapy (n=3 mice per group). After 14 and 21 days, mice were monitored with BLI for disease progression following intraperitoneal injection with D-luciferin (Goldbio, 150 mg/kg). All the mice were sacrificed when either experimental or humane endpoints were reached.

Statistical analysis

SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Data are expressed as the mean ± standard error and analyzed by t-text, with q-test method used for pairwise comparison. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of PD-1 on CD3$^+$ T cells and PD-L1 on DLBCL cells

The mean expression of PD-1 in the CD3$^+$ T cells isolated from the five healthy donors was 23.35±5.06%. The mean expression of PD-L1 was as follows: Pfeiffer (34.46±3.26%), U2932, OCI-ly7 and OCI-ly19 cells in three repetitions of the experiment (**Figure 1**).

Selection of E:T ratio, concentration of PD-1 inhibitor or PD-L1 inhibitor

The activity of T cells against Pfeiffer cells increased with the increase of the E:T ratio at 48 h (**Figure 2A**). The results were same as those for U2932, OCI-ly7 and OCI-ly19 cells (**Figure 2B**). In order to reduce the direct killing

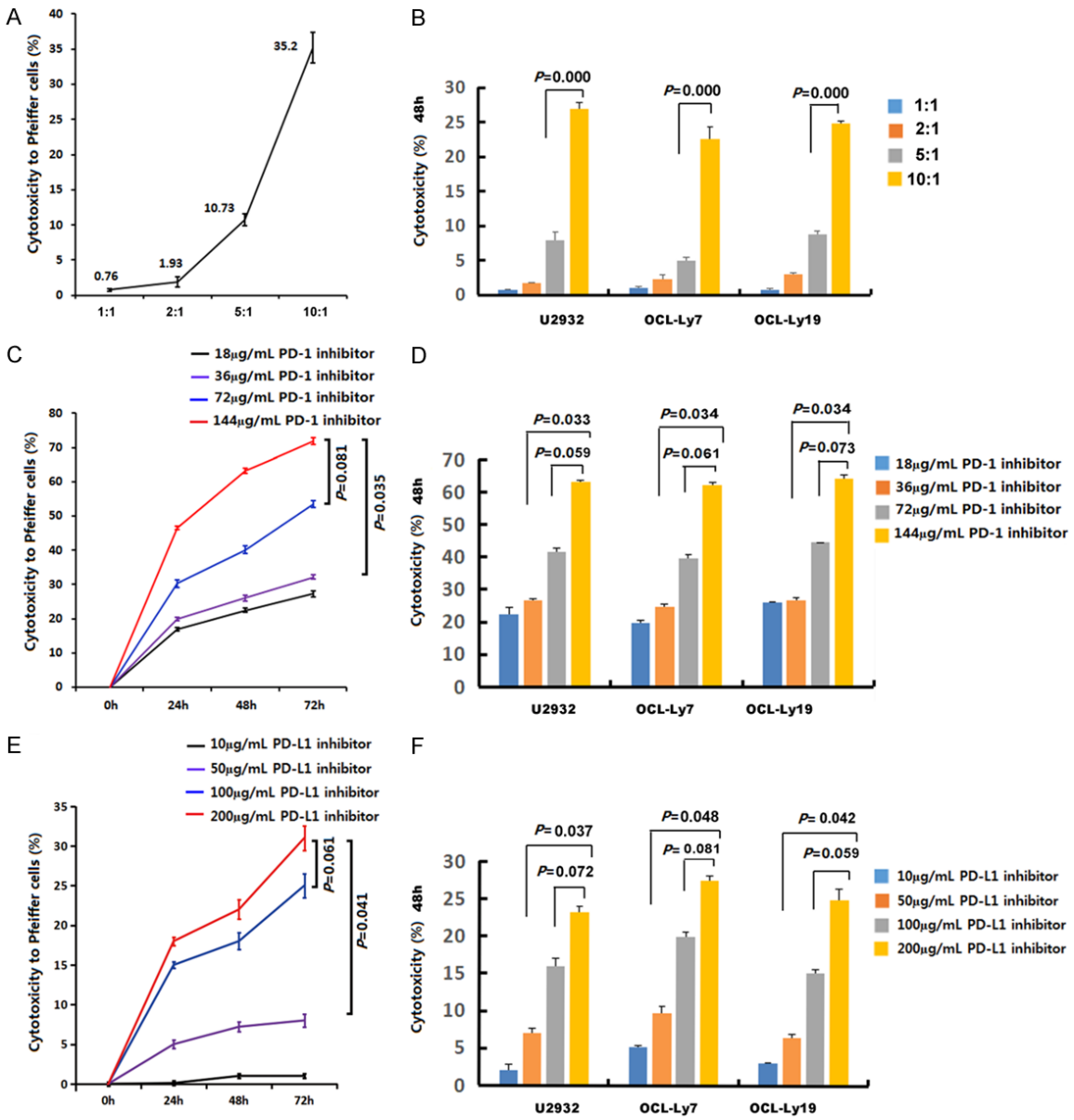
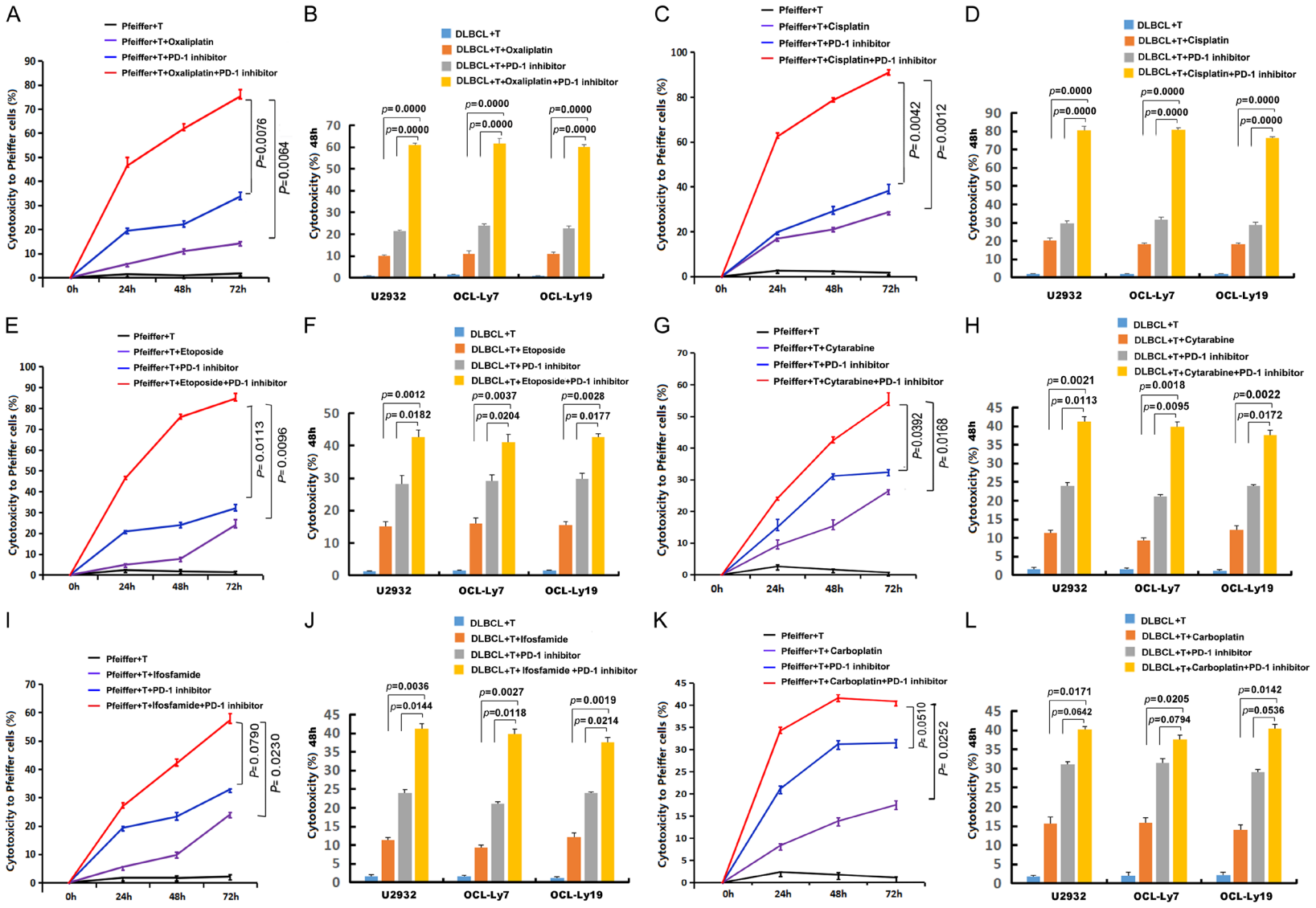


Figure 2. Selection of E:T ratio, concentration of PD-1 inhibitor or PD-L1 inhibitor. (A) The activity of T cells against Pfeiffer cells with the increase of the E:T ratio at 48 h. (B) The activity of T cells against U2932, OCI-ly7 and OCI-ly19 cells with the increase of the E:T ratio at 48 h. The activity of PD-1 inhibitor against Pfeiffer cells (C) and U2932, OCI-ly7 and OCI-ly19 cells (D). The activity of PD-L1 inhibitor against Pfeiffer cells (E) and U2932, OCI-ly7 and OCI-ly19 cells (F).

activity of T cells against DLBCL cell lines, the E:T ratio of 1:1 was selected for the following analysis of chemotherapeutic drugs combined with PD-1 inhibitor or PD-L1 inhibitor. The activity of PD-1 inhibitor or PD-L1 inhibitor against Pfeiffer cells increased with the gradually increasing doses of inhibitors (**Figure 2C** and **2E**). The results were same as those for U2932, OCI-ly7 and OCI-ly19 cells (**Figure 2D** and **2F**). We selected 36 μg/mL PD-1 inhibitor and 100 μg/mL PD-L1 inhibitor in the following synergistic experiments.

Cytotoxicity of PD-1 inhibitor combined with chemotherapeutic drugs on DLBCL cell lines

The cytotoxicity of PD-1 inhibitor combined with oxaliplatin, cisplatin and etoposide was significantly higher compared with that of the chemotherapeutic drug group ($P<0.01$; **Figure 3A-C**).


A
Pfeiffer+T
Pfeiffer+T+Oxaliplatin
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Oxaliplatin+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0076
P=0.0064
0h
24h
48h
72h
B
DLBCL+T
DLBCL+T+Oxaliplatin
DLBCL+T+PD-1 inhibitor
DLBCL+T+Oxaliplatin+PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0000
U2932
OCL-Ly7
OCL-Ly19
C
Pfeiffer+T
Pfeiffer+T+Cisplatin
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Cisplatin+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0042
P=0.0012
D
DLBCL+T
DLBCL+T+Cisplatin
DLBCL+T+PD-1 inhibitor
DLBCL+T+Cisplatin+PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0000
E
Pfeiffer+T
Pfeiffer+T+Etoposide
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Etoposide+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0113
P=0.0096
F
DLBCL+T
DLBCL+T+ Etoposide
DLBCL+T+ PD-1 inhibitor
DLBCL+T+ Etoposide+PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0012
p=0.0182
p=0.0037
p=0.0204
p=0.0028
p=0.0177
G
Pfeiffer+T
Pfeiffer+T+Cytarabine
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Cytarabine+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0392
P=0.0168
H
DLBCL+T
DLBCL+T+Cytarabine
DLBCL+T+PD-1 inhibitor
DLBCL+T+Cytarabine+PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0021
p=0.0113
p=0.0018
p=0.0095
p=0.0022
p=0.0172
I
Pfeiffer+T
Pfeiffer+T+Ifosfamide
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Ifosfamide+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0790
P=0.0230
J
DLBCL+T
DLBCL+T+Ifosfamide
DLBCL+T+PD-1 inhibitor
DLBCL+T+ Ifosfamide +PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0036
p=0.0144
p=0.0027
p=0.0118
p=0.0019
p=0.0214
K
Pfeiffer+T
Pfeiffer+T+Carboplatin
Pfeiffer+T+PD-1 inhibitor
Pfeiffer+T+Carboplatin+PD-1 inhibitor
Cytotoxicity to Pfeiffer cells (%)
P=0.0510
P=0.0252
L
DLBCL+T
DLBCL+T+Carboplatin
DLBCL+T+PD-1 inhibitor
DLBCL+T+Carboplatin+PD-1 inhibitor
Cytotoxicity (%) 48h
p=0.0171
p=0.0642
p=0.0205
p=0.0794
p=0.0142
p=0.0536

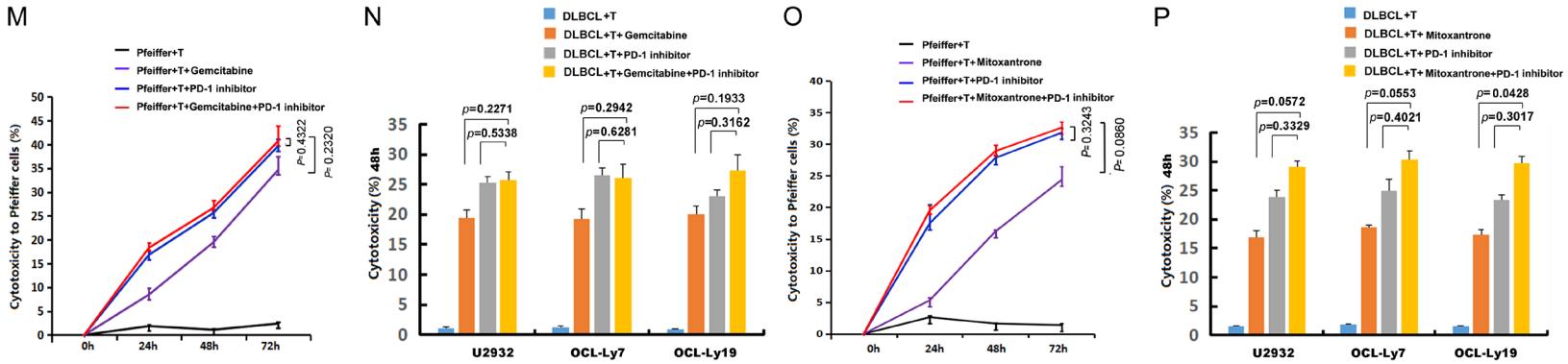


Figure 3. Cytotoxicity of PD-1 inhibitor combined with chemotherapeutic drugs on DLBCL cell lines. The cytotoxicity of PD-1 inhibitor combined with/or oxaliplatin (A, B), cisplatin (C, D) and etoposide (E, F). The cytotoxicity of PD-1 inhibitor combined with/or cytarabine (G, H), ifosfamide (I, J) and carboplatin (K, L). The cytotoxicity of PD-1 inhibitor combined with gemcitabine (M, N) and mitoxanthraquinone (O, P).

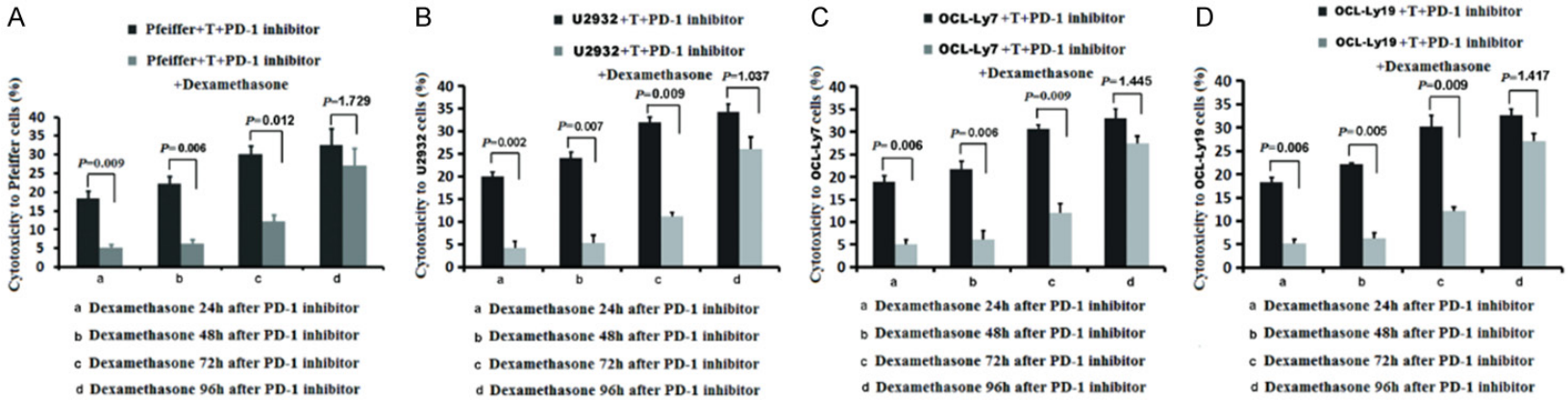


Figure 4. Effect of dexamethasone on the PD-1 inhibitor. The culture system included T cells and DLBCL cell lines (E:T=1:1), 36 μg/ml PD-1 inhibitor. Dexamethasone (0.08 mg/ml) was added to the culture system at 24, 48, 72 and 96 h. The cytotoxicity of T cells combined with PD-1 inhibitor against DLBCL cell lines by dexamethasone addition to the culture system at 24, 48 and 72 h groups (A-D).

The cytotoxicity of PD-1 inhibitor combined with cytarabine, ifosfamide and carboplatin was higher compared with that of the chemotherapeutic drug group ($P<0.05$; **Figure 3D-F**). However, there was no difference in the cytotoxicity of PD-1 inhibitor combined with gemcitabine and mitoxanthraquinone compared with the chemotherapeutic drug group ($P>0.05$, **Figure 3G** and **3H**). The cytotoxicity of PD-1 inhibitor combined with oxaliplatin and cisplatin was significantly higher compared with that of the PD-1 inhibitor group ($P<0.01$; **Figure 3A** and **3B**). The cytotoxicity of the PD-1 inhibitor combined with etoposide and cytarabine was higher compared with that of the PD-1 inhibitor group ($P<0.05$; **Figure 3D-F**). However, there was no difference in the cytotoxicity of the PD-1 inhibitor combined with ifosfamide, carboplatin, gemcitabine and mitoxanthraquinone with the PD-1 inhibitor group ($P>0.05$; **Figure 3G** and **3H**).

Effect of dexamethasone on the PD-1 inhibitor

The culture system included T cells and DLBCL cell lines (E:T=1:1), with 36 μg/ml PD-1 inhibitor. Dexamethasone (0.08 mg/ml) was added to the culture system at 24, 48, 72 and 96 h. The cytotoxicity of T cells combined with PD-1 inhibitor against DLBCL cell lines was inhibited by dexamethasone addition to the culture system at 24, 48 and 72 h groups and it was lower compared with that of the control group without dexamethasone at the same time point. However, there was no difference in cytotoxicity between the group of dexamethasone added at 96h and the group without dexamethasone at 96 h (**Figure 4**).

Cytotoxicity of the PD-L1 inhibitor combined with chemotherapeutic drugs on DLBCL cell lines

The cytotoxicity of PD-L1 inhibitor combined with cisplatin, oxaliplatin and etoposide in culture system of T cells and DLBCL cell lines (E:T=1:1) was observed. There was no difference in cytotoxicity between the groups with or without PD-L1 inhibitor (**Figure 5**).

Effect of chemotherapeutic drugs combined with PD-1 inhibitor in mice

In the tumorigenic model, all groups of mice displayed no serious toxicities and maintained an overall stable weight. The mice were monitored with BLI for disease progression. The DHAP regimen combined with PD-1 inhibitor (dexamethasone after 96 h of PD-1) group achieved the best curative effect in this study. As the selected dose of the DHAP/GemOx chemotherapy regimen was half of the clinically recommended dose, the mice in these two groups could not achieve the optimal curative effect from the therapy (**Figure 6**).

At 21 days of the mouse experiment, the PD-1 inhibitor alone group exhibited limited efficacy and slightly reduced tumor size. The reduction in the tumor size was more obvious in the DHAP combined with PD-1 inhibitor group (dexamethasone after 96 h of PD-1) compared with that of the DHAP group ($P=0.007$), the PD-1 inhibitor group ($P=0.001$) and the DHAP combined with PD-1 inhibitor group (dexamethasone after 24 h of PD-1) ($P=0.005$). However, there was no difference in the reduction of tumor size between the DHAP and the DHAP combined with PD-1 inhibitor (dexamethasone after 24 h of PD-1) groups ($P=0.573$). The reduction in the tumor size was more obvious in the GemOx combined with PD-1 inhibitor group compared with that in the GemOx group ($P=0.037$). There was no difference in the reduction of the tumor size between the GemOx and the PD-1 inhibitor groups ($P=0.983$; **Figure 7A**). The changes of the Avg radiance were consistent with the changes of the tumor size (**Figure 7B**). The survival time of mice in all the groups demonstrated that mice in the DHAP combined with PD-1 inhibitor (dexamethasone after 96 h of PD-1) and in the GemOx combined with PD-1 inhibitor groups had the longest survival compared with the mice in all other groups. The mice of DHAP combined with PD-1 inhibitor group (dexamethasone after 96 h of PD-1) all survived for 60 days after treatment (**Figure 7C**).

Discussion

DLBCL is the most common type of NHL [11]. The most widely used regimen is R-CHOP (rituximab + cyclophosphamide + doxorubicin + vindesine + prednisone) [12]. Chemotherapy is one of the main treatments for cancer, but the anttumor effect of chemotherapeutic drugs is affected by the patient's immune status. In clinical studies, chemotherapeutic drugs have a poor efficacy in patients with immuno-

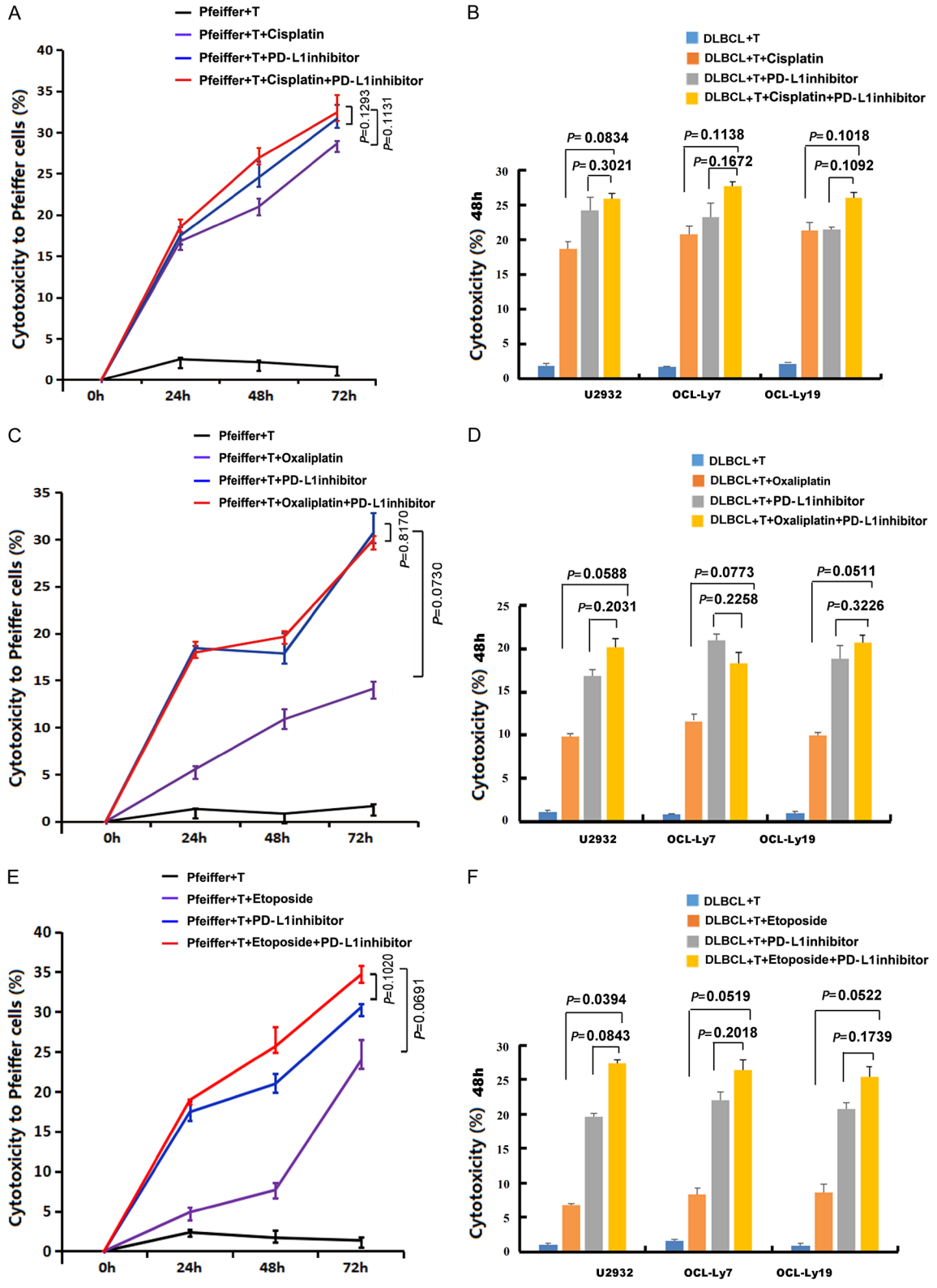


Figure 5. Cytotoxicity of the PD-L1 inhibitor combined with chemotherapeutic drugs on DLBCL cell lines. The cytotoxicity of PD-L1 inhibitor combined with/or cisplatin (A, B), oxaliplatin (C, D) and etoposide (E, F) in culture system of T cells and DLBCL cell lines (E:T=1:1) was observed. There was no difference in cytotoxicity between the groups with or without PD-L1 inhibitor.

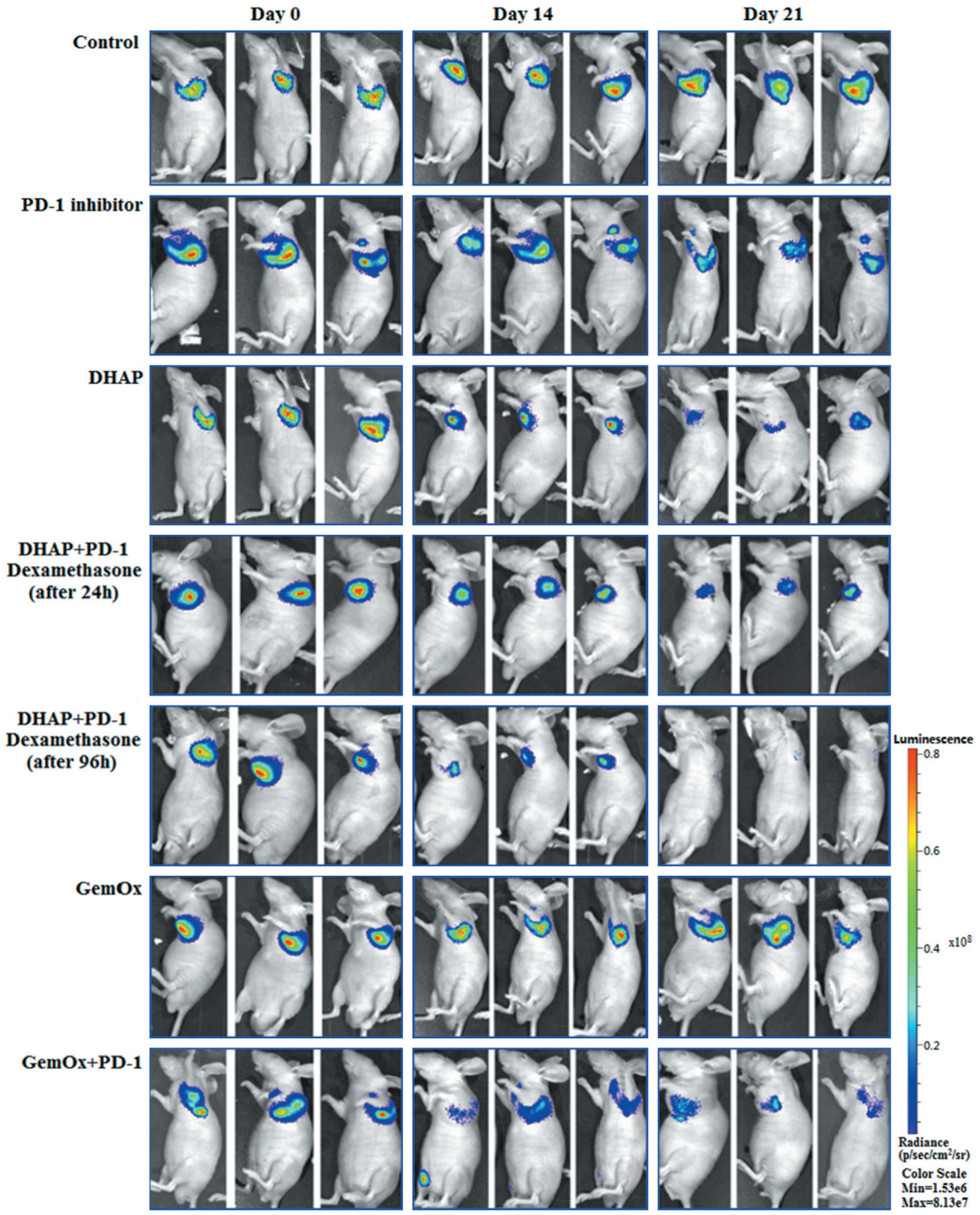


Figure 6. Effect of chemotherapeutic drugs combined with PD-1 inhibitor in mice. The mice were monitored with BLI for disease progression. The groups were divided into control group, PD-1 inhibitior group, DHAP regimen group, DHAP regimen combined with PD-1 inhibitor (dexamethasone after 24 h of PD-1) group, DHAP regimen combined with PD-1 inhibitor (dexamethasone after 96 h of PD-1) group, GemOx group and GemOx combined with PD-1 inhibitor group. The photo were showed in above groups at 0 day, 14 day and 21 day.

deficiency [13], suggesting that the antitumor effect of chemotherapy drugs may be improved by regulating the immune system of the patients.

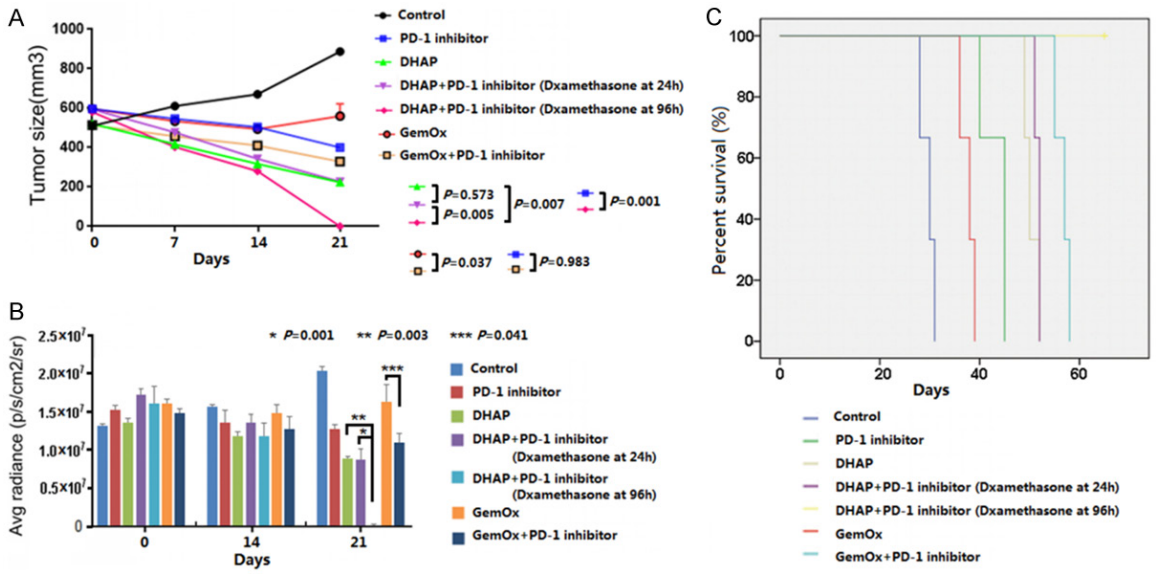


Figure 7. Effect of chemotherapeutic drugs combined with PD-1 inhibitor in mice. At the 21 days of this study in mice, mice were monitored for established tumors by bioluminescence imaging (BLI) in vivo. A. The tumor size were counted with above groups. B. The Avg radiance were counted with above groups. C. The survival rate were counted with above groups.

PD-1 expressed on T cells binds to PD-L1/L2 on tumor cells or other target cells, and then a downstream signaling pathway leads to the exhaustion of T cells and escape from immune surveillance of tumor cells [14]. PD-1 plays a key role in peripheral tolerance and homeostasis by inhibiting T-cell activation through interaction with PD-L1 expressed on tumor cells and non-malignant microenvironment cells (MECs) activating the checkpoint pathway associated with tumor evasion mechanism [15]. PD-1 inhibitors have been applied in a variety of solid tumors to prolong the OS and PFS [16-18]. In previous clinical trials, PD-1 inhibitors have shown therapeutic effects on hematological malignancies such as classic Hodgkin's lymphoma [19], follicular lymphoma [20] and DLBCL [21]. In recent years, the PD-1/PD-L1 axis has been shown to be correlated with the prognosis of patients with DLBCL [22].

In the chemotherapy group (pemetrexil + carboplatin) combined with PD-1/PD-L1, the effect was twice as much as that in the chemotherapy group, and the PFS was significantly prolonged in extensive-stage small-cell lung cancer [23]. It was previously demonstrated that chemotherapy used for the treatment of lymphoma patients has induced tumor immune evasion by upregulation of PD-L1 expression in bone marrow stromal cells [24]. According to the experimental results, PD-1 inhibitors performed better than PD-L1 blockers in terms of OS benefits, including total survival and PFS. In terms of drug safety, PD-1 inhibitors have relatively fewer side effects [25]. Although immune checkpoint blockade appeared to hold promise in the treatment of refractory DLBCL, the majority of the patients did not respond to single-agent PD-1 inhibitor [26]. PD-1 inhibitor combination with conventional chemotherapy exhibited shown synergistic efficacy through modifying the tumor microenvironment or releasing multiple tumor neoantigens. Agents such as anthracycline and platinum agents may lead to immunogenic cell death and trigger the uptake and regulation of tumor antigens [27, 28]. Therefore, the underlying mechanisms must be explored in depth, as chemotherapy combined with PD-1/PD-L1 inhibitors may prove to be an effective strategy for the treatment of lymphoma and other malignancies.

The present study demonstrated that PD-1 inhibitors combined with the certain chemotherapeutic drugs (cisplatin, oxaliplatin or etoposide) could significantly inhibit the proliferation of tumor cells *in vitro*. The synergistic effects of PD-1 inhibitors and cytarabine, carboplatin and isocyclophosphamide were weak-

er compared with those of the PD-1 inhibitors combined with cisplatin, oxaliplatin and etoposide. It was further observed that the synergistic effects of PD-1 inhibitors and chemotherapeutic drugs were superior to those of PD-L1. In the *in vivo* study, it was observed that the DHAP regimen combined with PD-1 inhibitor (dexamethasone after 96 h of PD-1) and the GemOx regimen combined with PD-1 inhibitor had satisfying synergistic effects. The DHAP regimen combined with PD-1 inhibitor (dexamethasone after 96 h of PD-1) exhibited an optimal tumor-suppressive efficacy in our study in mice.

Chemotherapy regimens for NHL often contain glucocorticoids. The glucocorticoids may affect the efficacy of immune checkpoint blockade within a short period [29]. Our results revealed that the synergistic effects of PD-1 inhibitor and chemotherapeutic regimens could not be inhibited by the glucocorticoid when it was added after 96 h of PD-1 inhibitor.

Therefore, the results of the present study revealed the synergistic effects of PD-1 inhibitor combined with chemotherapeutic regimens in Pfeiffer cells *in vitro* and *in vivo*. These results may prove to be of value in terms of curative effects in patients with R/R DLBCL. However, further studies are required to fully elucidate the underlying mechanisms and to provide evidence supporting the use of PD-1 inhibitors in polytherapy with chemotherapeutic regimens.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81900186, 81800105). Ethical approval and informed consent were obtained. Healthy donors agreed to participate this experiment as part of a clinical trial at the Department of Hematology at Tianjin First Central (Tianjin, China) hospital with autologous CAR-T 19 cells (ChiCTR-ONN-16009862). All animal procedures were approved by the institutional animal and care use committee of Tianjin First Central Hospital (Tianjin, China).

Disclosure of conflict of interest

None.

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