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LETTER TO THE EDITOR

Diverse effects of chemotherapeutic agents on immune cell function and implications in immunochemotherapy

Dear Editor,

Immunotherapy, such as the use of immune checkpoint blockade antibodies, has revolutionized cancer treatment resulting in a significant improvement of clinical outcome [1, 2]. As chemotherapeutic agents are still the major therapeutic modalities, their combination with immunotherapy is emerging as a new multi-target strategy to eliminate cancer [3]. However, it is still unclear which chemotherapeutic agents are most suitable for combination with immunotherapy. In this context, our goal was to determine which chemotherapeutic agents could stimulate or inhibit T cell functions and thus could potentially be used as a guide in selecting drugs for combination with immunotherapy to improve cancer treatment outcomes.

In order to investigate the effect of chemotherapies on T cell function, we have recently adapted a T cell activation assay suitable for the evaluation of multiple drugs [4]. Using this assay, we have investigated the effect of 23 "classical" chemotherapeutic agents. The drugs were divided into 6 groups: (1) DNA alkylating agents (chlorambucil, melphalan, carmustine, busulfan, dacarbazine, and temozolomide); (2) platinum drugs (cisplatin, carboplatin, and oxaliplatin); (3) antimetabolites and DNA incorporating agents (fludarabine, 5-fluorouracil, cytarabine, gemcitabine, and methotrexate); (4) topoisomerases inhibitors (daunorubicin, doxorubicin, etoposide, camptothecin, and mitoxantrone); (5) microtubule-targeting agents (vinblastine, paclitaxel, and docetaxel); (6) molecules classified as "others" (arsenic trioxide). The mechanisms of action of these drugs are rather diverse [5]: DNA alkylating agents form DNA crosslinks and inhibit DNA synthesis and function. Platinum salts form intrastrand and interstrand crosslinks and also bind covalently to proteins. Antimetabolites inhibit DNA synthesis, repair, and function. Moreover, they incorporate into DNA and induce apoptosis. The topoisomerase inhibitors listed are mainly topoisomerase II inhibitors while camptothecin is a topoisomerase I inhibitor. Some of them could also induce DNA damage, intercalate to DNA, inhibit DNA and RNA synthesis, or generate reactive oxygen species (ROS). The members of the microtubule-targeting agents inhibit mitosis by altering the microtubule assembly or tubulin polymerization. Arsenic trioxide induces ROS generation, inhibits cell proliferation, promotes apoptosis, stimulates differentiation, and binds to thiol groups of proteins [6].

The cancer cells used in this T cell activation assay was B16 mouse melanoma cells expressing ovalbumin (B16-OVA). This cell model permits us to test whether the drugs impact the functions of immune cells. Since the established cancer cell lines might have natural sensitivity or resistance to chemotherapeutic agents, a major issue was to choose the proper concentrations of the different drugs for use in this assay. For example, we found that B16-OVA cells were very sensitive to gemcitabine and daunorubicin with IC50 values of 21 nmol/L and 22 nmol/L, respectively. These concentrations are much lower than the drug concentrations in a patient's plasma (89.3 μmol/L and 0.31 μmol/L, respectively) [7]. Considering that the plasma drug concentrations of the treated patients do not always reflect the drug concentrations observed in the tumor microenvironment, we decided to incubate the cancer cells with all the drugs at IC25 concentrations to compare their effect on the immune cells. We initially performed a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay for cell proliferation after incubating B16-OVA cells with various concentrations of drugs for 48 h to access their IC25 concentrations (Figure 1A). The cytotoxic curves for each drug are shown in

List of abbreviations: ICD, immunogenic cell death; HMGB1, high mobility group box 1; ATP, adenosine triphosphate; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; Cmax, maximum plasma concentration; BMDC, bone marrow dendritic cell; TNFα, tumor necrosis factor-alpha; IFNγ, interferon-gamma; IL-2, interleukin-2; PI, propidium iodide; ELISA, enzyme-linked immunosorbent assay; ATCC, American Type Culture Collection; PBS, phosphate-buffered saline; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; DMSO, dimethyl sulfoxide; CAS No., chemical abstract service registry number; IC25, drug concentration that kills 25% of cancer cells; IC50, drug concentration that kills 50% of cancer cells; N/A, not applicable; SD, standard deviation; SEM, standard error of the mean

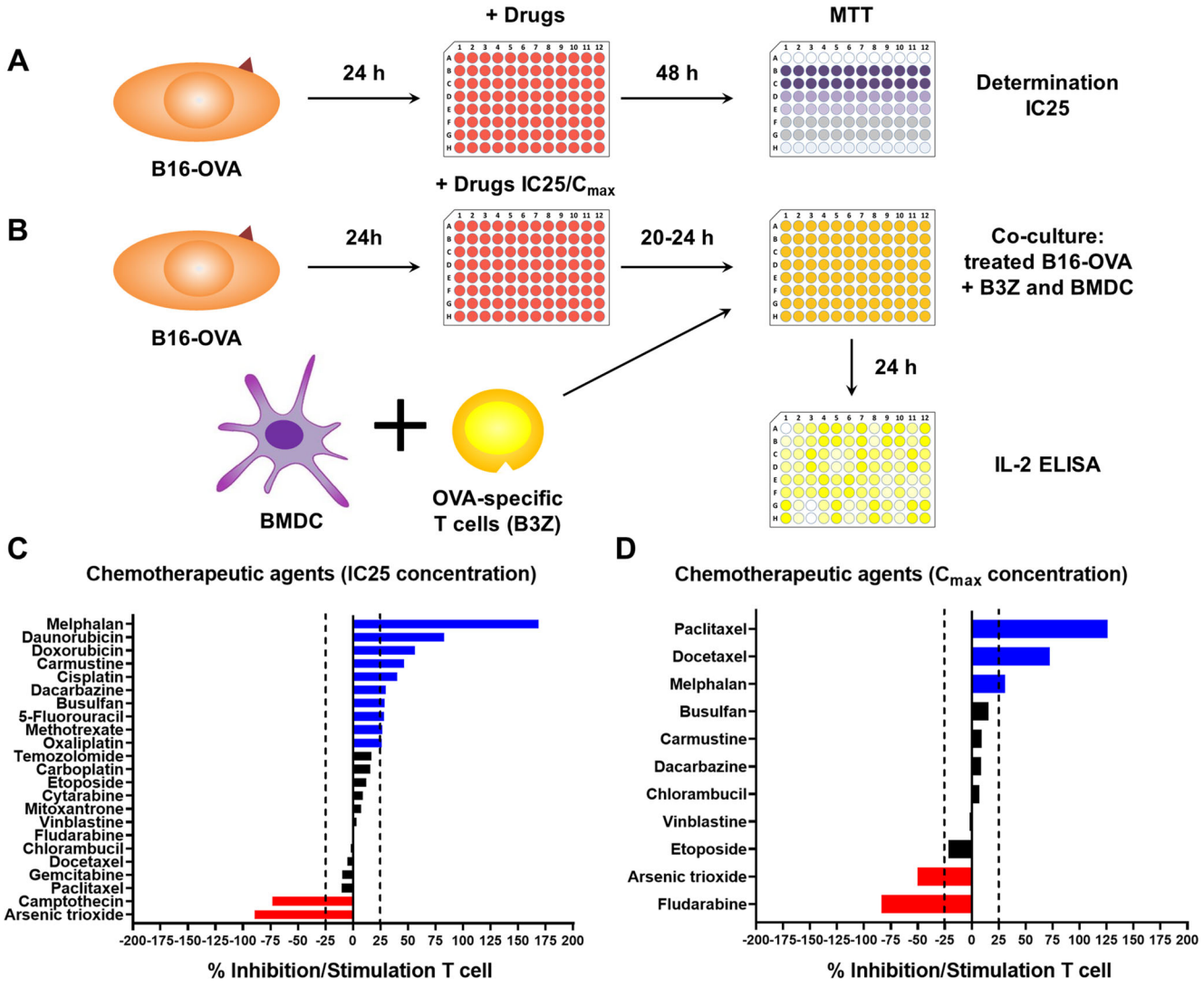

FIGURE 1 Experiment design of the T cell-based drug screening and list of drugs that stimulate or inhibit T cell function. A. Determination of IC25 concentrations of the 23 selected chemotherapeutic agents was determined by MTT assay after 48-h incubation in B16-OVA cancer cells. B. Determination of T cell function when B16-OVA cancer cells and immune cells (BMDC + B3Z) were exposed to the drugs. Detailed protocols are provided and summarized in Materials and Methods section in the Supplementary files. C-D. List of chemotherapeutic drugs (at IC25 or C_{max} concentration) that modify the secretion of IL-2 levels reflecting T cell functions. The data are expressed by percent of increase (stimulation) or decrease (inhibition) compared to untreated B16-OVA cells. A change of 25% in IL-2 levels, compared to untreated cells, was used as a cut-off value to determine the stimulatory or inhibitory effect on immune function. Blue color represents drugs that stimulate immune function. Red color represents drugs that inhibit immune function. Black color represents drugs that does not modify immune function. Abbreviations: BMDC, bone marrow dendritic cells; C_{max}, maximum plasma concentration of the drug quantified in patients; ELISA, enzyme-linked immunosorbent assay; IC25, drug concentration that kills 25% of cancer cells; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

Supplementary Figure S1 and the respective IC25 concentrations are indicated in Supplementary Table S1. For cytostatic drugs (chlorambucil, temozolomide, paclitaxel, and docetaxel), the IC25 concentrations were manually determined. In parallel, the cells were also exposed to 11 drugs at maximum plasma concentrations (C_{max}), according to the previous study [7].

In order to evaluate the stimulatory or inhibitory effect of chemotherapeutic agents on immune cell functions, we used a T cell activation assay, shown in Figure 1B. Briefly, B16-OVA melanoma cells were first pre-treated with drugs at IC25 or C_{max} concentrations for 24 h to induce changes that could potentially inhibit or stimulate immune cells. The treated cancer cells were then cultured with OVA-specific T cells (B3Z) and bone marrow dendritic cells (BMDCs) for an additional 24-h incubation. The drug-containing medium was not removed in order to mimic the real situation of a tumor microenvironment and to observe the direct effect of these drugs on immune cells. Dendritic cells were used as antigen-presenting cells

to present protein antigens to the T cells. B3Z cell line is a $CD8^+$ T-cell hybridoma engineered to detect a peptide derived from chicken ovalbumin [8], which was expressed by B16-OVA cancer cells. Upon stimulation, the T cells would secrete pro-inflammatory molecules including tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), and interleukin-2 (IL-2). To quantify the stimulation of T cells, we measured IL-2 levels in the culture medium as a readout. A change of 25% in IL-2 levels was used as a cut-off value to determine the stimulatory or inhibitory effect on T cell function as shown in Figure 1C-D and Supplementary Table S2. The raw data of IL-2 levels for each drug are shown in Supplementary Figure S2. The relevant Materials and Methods are summarized in the Supplementary files.

Supplementary Table S2 shows that melphalan, daunorubicin, and doxorubicin, at IC25 concentrations, had the best ability to stimulate T cells. The family of DNA alkylating agents exhibited the most stimulatory molecules. Indeed, four out of the six compounds showed stimulatory effects on T cells (Supplementary Table S2 and Supplementary Figure S2A). Arsenic trioxide and camptothecin were the two drugs that decreased the IL-2 levels (Supplementary Table S2 and Supplementary Figure S2A).

At C_{max} concentration, although lower than the IC25 concentration, arsenic trioxide still caused a decrease in IL-2 secretion (Supplementary Table S2 and Supplementary Figure S2B), while melphalan still stimulated T cell function (Supplementary Table S2 and Supplementary Figure S2B). The other members of alkylating agents did not cause significant changes at lower concentrations. Interestingly, paclitaxel and docetaxel strongly enhanced T cell function at 4.27 μmol/L and 5.47 μmol/L, respectively (Supplementary Table S2 and Supplementary Figure S2B), which corresponded to approximately 50% cell death after 48-h incubation (**data not shown**). Based on these findings, the drug-mediated effect on T cell stimulation could not be simply explained by one specific mechanism.

To explain the immunosuppressive effects of camptothecin, fludarabine, and arsenic trioxide, we explored their cytotoxic effects on mouse T cells. B3Z cells were exposed to stimulatory drugs (IC25: melphalan, daunorubicin; C_{max}: paclitaxel, docetaxel) and immunosuppressive chemotherapies (IC25: camptothecin, arsenic trioxide; C_{max}: fludarabine, arsenic trioxide) at their IC25 and C_{max} concentrations for 24-h. Cell death was then determined by flow cytometry after stained with propidium iodide (PI) and Annexin V. Although melphalan was the best stimulatory molecule, it exhibited some T cell toxicity (Supplementary Figure S3A-B). Meanwhile, daunorubicin did not show much toxicity (Supplementary Figure S3A-B). Camptothecin and arsenic trioxide showed a severe T cell cytotoxicity (Supplementary Figure S3A-B), thus explaining strong inhibitory effect when cells were exposed to these two drugs (Supplementary Table S2 and Supplementary Figure S2A). Melphalan, paclitaxel, and docetaxel (at C_{max} concentration) displayed certain cytotoxicity (Supplementary Figure S3C-D). While arsenic trioxide (at C_{max} concentration) enhanced T cell death, fludarabine showed little T cell toxicity (Supplementary Figure S3C-D). Arsenic trioxide and fludarabine, at low concentrations, seemed to have direct effect on T cell function by altering the secretion of cytokines such as IL-2 or IFN-γ. Interestingly, fludarabine did not alter IL-2 secretion when used at higher concentrations (Supplementary Table S2 and Supplementary Figure S2A). Indeed, the IC25 concentration (20.2 μmol/L) was about 6-fold higher than C_{max} concentration (3 μmol/L) for this drug. This chemotherapeutic drug might be immunosuppressive or immunostimulant depending on its concentration.

Considering these results, melphalan might be an interesting choice in the context of immunochemotherapy. Daunorubicin and doxorubicin also showed promising effects on T cell stimulation. However, B16-OVA cells are very sensitive to these two drugs *in vitro*. Further studies with other cell lines might be necessary to show the general impact of these two drugs on T cell function. Since paclitaxel and docetaxel were cytostatic in this cellular model, the choice of the drug concentration might be inadequate. If the plasma concentration is an indication of the clinical situation, these two chemotherapeutic agents are therefore potential drugs to combine with immunotherapies. Indeed oxaliplatin, cyclophosphamide, doxorubicin, and paclitaxel have already demonstrated the ability to potentiate the efficacy of immunotherapies [9]. Further *in vivo* experiments and clinical studies are needed to validate the therapeutic relevance of this drug screening-based T cell activation assay. Finally, our findings also provide a cautionary note against using drugs such as camptothecin, arsenic trioxide, and fludarabine, in combination with immunotherapy due to their strong immunosuppressive effect.

DECLARATIONS

Ethics approval and consent to participate.

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The key raw data have been deposited into the Research Data Deposit (http://www.researchdata.org.cn), with the

Approval Number of RDDB2020001022 and the datasets used in this study are publicly available.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

C.G., P.Z., and L.C. performed experiments. C.G., X.X., and P.H. analyzed, interpreted the data and wrote the paper. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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