Combined ROS-related therapy improves the efficacy of multiple neoantigen vaccines against LUAD

Peijia Lai, Tianyu Cui, Zhiheng Zhang

Abstract

Lung adenocarcinoma (LUAD) is classified as Non-Small-Cell Lung Cancer (NSCLC), one of the most malignant cancers due to its poor prognosis and fast progression. Its resistance to chemotherapy, immunosuppressive tumor microenvironment (TME), and frequent immune evasion also impose enormous obstacles in treating LUAD. However, previous research found that these tumor cells resistant to chemotherapies commonly increase their production of intracellular reactive oxygen species (ROS) due to high mitochondria activity. Antioxidants like glutathione (GSH) are also highly produced to maintain their redox homeostasis. Therefore, the depletion of antioxidants which can lead to intratumoral accumulation of ROS to cytotoxic levels, provides a new therapeutic target. Besides, the high level of extracellular ROS also significantly contributes to the immunosuppressive characteristic of TME, which exhausts activated T cells. This work provides the framework for a novel combinational therapy based on a multiple-neoantigen vaccine to avoid immune evasion. Two types of nanoparticles further boost the vaccine's efficacy against tumors, one depleting intratumoral GSH and scavenging extracellular ROS. Anticipated results are shown from various aspects, hoping the innovative treatment can effectively improve the survival rate with few side effects.

Keywords: Lung Adenocarcinoma, Multiple neoantigen vaccines, tumor microenvironment, reactive oxygen species, Intratumoral GSH depletion, TME ROS scavenging

I. Introduction

A. Lung adenocarcinoma (LUAD): a highly resistant cancer that is difficult to diagnose and treat

Lung cancer is the second most prevalent cancer responsible for around 10% of cancer cases worldwide[1]. In addition, lung cancer has the worst prognosis among all types of cancers, with a 5-year survival rate of only around 10% [2]. LUAD belongs to Non–Small-Cell Lung Cancer (NSCLC), a subtype other than Small-Cell Lung Cancer. LUAD is the most common, accounting for almost half of all lung cancers[3]. Apart from its high frequency, most LUAD is diagnosed in an advanced state which means traditional radiotherapy, surgery, and chemotherapy are often inadequate for treatment[4]. LUAD will also quickly develop resistance to vast kinds of chemotherapies which makes treatment even more difficult[5]. For those reasons, a new combined innovative immunotherapy is needed.

Interestingly, almost all resistant tumor cells show increased ROS production due to elevated mitochondria activity which can be a potential target for new immunotherapy. Additionally, lung cancers always have a very high mutational burden and only one-third of the neoantigens are present on every tumor[6]. Consequently,

there is a need to select and present multiple neoantigens to avoid antigen escape and antigen depletion. Since NAD[P]H: quinone oxidoreductase (NQO1) is expressed abundantly in NSCLC, it has been considered a target to selectively deliver drugs [7].

This study will be focusing on the high extracellular and intracellular ROS in lung cancer and their role in promoting tumor growth and forming immunosuppressive TME to enhance the efficacy of the neoantigen vaccine. By exploring the effects of intracellular ROS homeostasis disruption and extracellular ROS scavenging on the survival and immune evasion of the tumors, this study thus proposes a novel hypothetical treatment for LUAD.

B. Multiple-neoantigen vaccine: personalized peptide-based vaccine

Traditional therapies like chemotherapy showed poor potency due to low immunogenicity, so it is necessary to develop peptide-based vaccines with higher antigen specificity. Neoantigens are new antigens made by the unique nonsynonymous mutations of the tumor cells and provide a promising target for immunotherapy. Research has confirmed that personalized peptide neoantigen vaccines, which present neoantigens to dendritic cells (DCs) and induce T cell response, are capable of treating various types of cancer[8].

Neoantigens, i.e. self-proteins that carry mutations that

are unique to the tumor cells can be identified fairly rapidly through whole-exon sequencing approaches. It is then imperative to test whether peptides harboring tumor-specific mutations can be presented on the HLA haplotypes of a given patient. Subsequently, it needs to be tested whether these peptides are indeed immunogenic and can provoke a tumor-specific immune response[9].

Tumors also frequently downregulate the expression of neoantigens to evade the immune system[6]. Delivery of multiple neoantigens through e.g. ferritin nanoparticles provides a potential strategy to solve the problem of antigen depletion. It has been proven that multiple neoantigen vaccines can more effectively activate DCs to induce vigorous T cell responses and control tumor growth[10]. In addition, studies have also demonstrated that the neoantigen vaccines are effective on elicit functional memory T cells. [11].

However, the efficacy of the neoantigen cancer vaccine is still limited because the highly immunosuppressive TME will inhibit the normal function of activated T cells. This work will discuss the role of TME in the following sections.

C. High extracellular ROS contribute to immunosuppressive TME

Reactive oxygen species (ROS) is a group of reactive oxygen-containing chemicals produced inevitably in mitochondria as a byproduct of respiration[12]. TME comprises tumor cells, tumor stroma cells, blood vessels, extracellular matrix, infiltrating inflammatory cells, fibroblasts, and various other tissues, supporting tumor growth, and inhibiting immune response. High levels of extracellular ROS produced by cancer cells significantly contribute to the immunosuppressive TME for the following reasons.

Firstly, a high global level of ROS increases the oxidative stress of the T cell and causes decreased ROS levels inside the T cells which are crucial for the signal transduction of TCR, so T cells cannot be activated[13].

Secondly, high global ROS will induce T cells to differentiate into regulatory T cells and activate myeloid-derived suppressor cells which together suppress immune reactions[14]. Some ROS can also stimulate the expression of Fas ligand (FasL) which mediates activation-induced cell death in T cells. High expression of Programmed death-1 (PD1) – an inhibitory receptor expressed on T cells - is also related to ROS[15].

Third, high ROS reprograms the cancer-associated fibroblasts and causes them to differentiate into myofibroblasts which further promotes the growth of the tumors[16].

Since the high level of extracellular ROS in TME contributes to immune cell dysfunction and tumor progression, lowering extracellular ROS levels can further boost immune response. This work will discuss the potential treatment targeting extracellular ROS levels in the approach segment.

D. Tumor cells producing high intracellular ROS for growth and metastasis are more vulnerable to antioxidant depletion

Cancer cells not only produce a high level of ROS into extracellular TME, but they also maintain high intracellular ROS. ROS also plays an important role in the regulation of metabolic activity, protein modification, transcription factors activities, and signal transduction[17]. However, at the same time, ROS are capable of damaging cellular protein and DNA, so normal cells steadily maintain a low level of intracellular ROS by producing antioxidants[14]. It has been postulated that cancer cells maintain high levels of ROS for the following reasons.

First, ROS stabilizes hypoxia-inducible transcription factors (HIFs) which up-regulate the expression of vascular endothelial growth factor (VEGF) and cause angiogenesis around the tumor to supply oxygen and nutrients.

Second, ROS activates the NF-kB pathway of the cell which releases TNF-a, other pro-inflammatory cytokines, and growth factors. Tumor cells depend on those to proliferate[18].

Third, high ROS promotes metastasis by improving cell adhesion, migration, and resistance to anoikis when they detach from the tissue and travel through the bloodstream[12].

However, cancer cells have to produce high levels of antioxidants in order to protect themselves from oxidative stress. Oxidative stress occurs when the balance between antioxidants and ROS is disrupted[19]. Increased oxidative stress will cause the mitochondria to release cytochrome C, initiating a series of intrinsic signals for apoptosis.

Therefore, depleting antioxidants to disturb tumor intracellular redox homeostasis is a promising cancer therapy. GSH is an important antioxidant to detoxify excessive ROS[20]. It reacts with ROS to form glutathione disulfide (GSSG). Cancer cells produce a high amount of GSH to detoxify high levels of ROS. Depletion of GSH will cause the accumulation of ROS in tumor cells to a cytotoxic level. Therefore, the depletion of GSH specifically in tumor cells has become another target to improve the efficacy of the multiple-neoantigen vaccines.

Several studies that outline novel immunotherapeutic approaches against LUAD are discussed in the following section. The chosen papers have highlighted the

significance of reducing the extent of immunosuppression in TME and depleting tumor intracellular GSH to boost the efficacy of multiple-neoantigens vaccines.

II. Summaries of primary research papers

A. Peptide-based neoantigen vaccine suppresses tumor growth in advanced pancreatic cancer[9].

This paper is a clinical analysis of the efficacy of peptide neoantigen vaccine in advanced cancer when patients have experienced metastasis. Although only one of seven patients who received the vaccine survived, all of them had experienced T cell priming and most of them showed reduced tumor growth or even a reduction in tumor volume. ELISpot assay revealed that five of the patients had an increase in IFN-γ, indicating the activation of T cells. Moreover, the results also suggested the differentiation of T cells since effector memory T cells were detected in patients.

B. Targeted scavenging of extracellular ROS relieves suppressive immunogenic cell death [21]

In this paper, the authors revealed the problem that immunogenic cell death (ICD) of tumors and the vitality of tumor-infiltrating T lymphocytes are severely weakened by increased ROS in TME. Therefore, the authors designed a ROS nanoscavenger masked by pH-sensitive polyethylene glycol (PEG) to target the tumor extracellular matrix (ECM). The nanoscavenger anchors on the acidic TME with pH 6.8 to eliminate the surrounding ROS away, so it can relieve the immunosuppressive ICD elicited by specific chemotherapy and prolong the survival of T cells. After the tumor cell death and the release of antigen to DCs, the antigen presentation in DC to CD8+T cells is also enhanced due to the decreasing ROS in TME. This therapy is highly specific targeting tumor cells with few side effects.

C. Tumour Depletion of GSH Causes Accumulation of Oxidative Stress [22]

In this paper, the authors tried to eliminate tumors by amplifying intracellular oxidative stress through the Fenton reaction and GSH depletion. Fenton reaction converts endogenous H2O2 to highly toxic hydroxyl radicals (·OH) that can cause irreversible oxidative damage to tumor cells. This method is known as chemodynamic therapy (CDT). However, the over-expression of antioxidant GSH in tumor cells can efficiently scavenge the toxic hydroxyl radicals so the therapeutic effect of Fenton reaction is severely limited. To overcome this challenge, the authors designed novel iron oxide nano-carriers loaded with beta-lapachone (Lapa) drugs which have both Fenton-like agents and GSH depletion properties. The Lapa can selectively increase tumor-site-specific generation of H2O2 via NAD(P)H: NQO1 catalysis. The iron ions released in the acidic TME can selectively convert H_2O_2 to hydroxyl radicals. Also, the NAD(P)H which acts as a coenzyme of GSH reductases is depleted during the reaction with NQO1, so the reduction of GSSG into GSH is inhibited. This work remarkably enhanced tumor-specific chemodynamic therapy with minimal side effects.

Based on these three primary research papers, this work hypothesizes that extracellular scavenging of ROS and intracellular depletion of GSH can inhibit tumor growth and create a more immunogenic TME. The two strategies work in synergy to further improve immune response and enhance the efficacy of multiple-neoantigens vaccines targeting LUAD.

III. Approaches

A. Methods and materials

This work proposed to transplant wild-type mice with syngeneic tumors as the model for LUAD, and different combinations of therapies are tested in vivo. All mice will receive tumor transplantation. After treatment, some mice tumor cells are also extracted for further in vitro research. In every experimental cohort, the tumor volume will be measured at day 10 and all of them will be sacrificed on day 30. Tumors are then removed and used for subsequent analysis.

B. General Approach

A series of experiments will be performed on 12 groups of healthy wild-type C57BL/6 mice. The details are illustrated in table 1.

Table 1. Groups of mice for experiment.

No	Group Type	Treatment	Intention
1	Control Group	Without any treatment	To serve as a benchmark
2	Control group	Inject vaccine without the neoantigens (e.g. ferritin, buffer solution)	To ensure that other components of the vaccine will not influence the immune response

No	Group Type	Treatment	Intention
3	Treatment Group	Inject vaccine with single neoantigen	To investigate whether multiple neoantigens are more effective in triggering immune response
4	Treatment Group	Inject vaccine with multiple neoantigens	
5	Treatment Group	Receive T cells from mice surviving tumor implantation in group1, 3, and 4 respectively. This group of mice has normal immune functions and is immunologically compatible with the donors.	To prove that the immune response stimulated by the vaccines has memory and is T-cell dependent. Also to prove that multiple neoantigens are most effective.
6	Treatment Group	Inject nanoparticles depleting intracellular GSH	To investigate the efficacy of the nanoparticles to deplete GSH and its efficacy of tumor suppression
7	Treatment Group	Inject nanoparticles depleting intracellular GSH. The mice are depleted with CD4+ and CD8+	To prove that depletion of GHS can directly cause apoptosis and act independently of T cells
8	Treatment Group	Inject nanoparticles reducing extracellular ROS in TME	To investigate the efficacy of the nanoparticles to reduce ROS in TME and its efficacy of tumor suppression
9	Treatment Group	Both kinds of nanoparticles are delivered	To test the efficacy of different combinations of immunotherapy.
10	Treatment Group	Nanoparticles Depleting Intracellular GSH with multiple-neoantigen vaccine	
11	Treatment Group	Nanoparticles Reducing Extracellular ROS in TME with multiple-neoantigen vaccine	
12	Treatment Group	Combination of the three therapies	

C. Mice Model and animal care

In this work, pathogen-free wildtype C57BL/6 mice at the age of 6 weeks with mixed genders are used to conduct experiments. All mice will have full access to a standard laboratory diet and water ad libitum. The mice will be nurtured on a controlled temperature and a 12 h light and dark cycle. All experimental procedures will strictly follow animal care guidelines.

D. Tumor Cell Line

X577 lung adenocarcinoma mouse cell lines will be used for this experiment[23]. X577 cells will be cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, antibiotics, and kept under 37 degrees Celsius with 5% CO2. They will be tested for the absence of mycoplasma routinely.

E. Orthotopic Intrapulmonary Implantation of X577 cells

5×10^5 X577 cells will be suspended in 50% HBSS/50% Matrigel and subcutaneously injected into the right flanks of each 6-week-old wild-type mouse. Tumor growth will be measured every day and they will be allowed to grow for 30 days. After 30 days, all surviving animals will be sacrificed by intravenous injection of potassium chloride and their tumor will be taken out.

F. Preparation of Vaccine and Purification

To produce a neoantigen, whole exome sequencing will be performed to identify the mutations and bioinformatics analysis will be conducted to test the affinity for MHC molecules. Five neoantigens will be selected according to MHC-I IC50 ≤ 500 nM, MHC-II binding score ≤1, minor allele frequency (MAF) ≥ 25%, and mutated RNA read ≥ 1[10]. Moreover, for delivery of cancer vaccines, ferritin will be introduced with a point mutation to remove a site for N-linked glycosylation. Proteins for immunization will be further purified before use. The vaccine is injected subcutaneously into mice.

G. Preparation and Delivery of Nanoparticles Reducing Extracellular ROS in TME

The synthesized chemical PEI-PPS self-assembles into the nanoscavengers (NS). The NS is then modified with Extracellular Domain (ECM) targeting peptides and are masked with pH-sensitive PEG capsules. The modified NS are injected intravenously into mice and the PEG capsules de-shield when they anchor to the acidic ECM in TME. The PEI-PPS then reduces the extracellular ROS by neutralizing them[21].

H. Preparation and Delivery of Nanoparticles Depleting Intracellular GSH

The nanoparticles depleting intracellular GSH are injected intravenously. This type of Fe3O4 nanoparticles is bonded with protocatechuic acid (PA), and human serum albumin (HSA). Then, the Lapa drugs which can specifically inhibit the intracellular GSH are encapsulated in the HSA. Finally, the iron oxide nanoparticles which can self-disassemble after delivery to release Lapa drugs are generated. The nanoparticles can penetrate tumor sites specifically due to the EPR (Enhanced Permeability and Retention) effect[22].

I. Combination of all three therapies

Prior to the vaccine, nanoparticles depleting intracellular GSH and nanoparticles detoxifying extracellular ROS are injected together intravenously to induce tumor apoptosis and relieve the immunosuppressive microenvironment. Then, the vaccine with better efficacy is injected subcutaneously into mice.

J. Depletion of CD4+ and CD8+ cells in the group treated with GSH Depletion

Prior to the GSH depletion, αCD4, and αCD8 antibodies are given intravenously to Group 6 mice to deplete their CD4+ and CD8+ cells. In this way, whether the depletion of GSH itself can still induce apoptosis of cancer cells independent of immune cells can be investigated.

K. Flow Cytometry Analysis of Lymphocyte Population Activated by Vaccines

The tumors are removed from the mice and a single cell suspension is prepared. The single cells in the suspension are then stained by fluorescent-labeled antibodies. Flow cytometry is used to identify their cell surface markers. The frequencies of Foxp3+ regulatory T cells, CD8+ T cells, and CD4+ T cells are thus calculated.

L. Flow Cytometry Analysis of Tumor Cell Apoptosis
Cancer cells that die of apoptosis can be stained with Annexin V-FITC and they can be counted directly through flow cytometry.

M. Enzyme-Linked Immunosorbent Assay (ELISA) to Measure the Level of Inflammatory Cytokines Produced by Activated Lymphocytes.

The serum will be collected 6 hours after injection and IFN-$_\beta$ concentration will be measured using VeriKine Mouse Interferon Beta ELISA Kit to assess the level of activated T cells.

N. T-cell proliferation assay CCK8

The CCK-8 (Cell Counting Kit-8) assay will test T cell proliferation. Peripheral blood mononuclear cells (PBMCs) from mice administered with multiple-neoantigen vaccines and single-neoantigen vaccines will be seeded into 96-well plates. Then,10 μl of CCK-8 solution will be added to each well and the plate will be incubated at 37 degrees Celsius for 2 hours. Finally, absorbance at 450 nm will be measured. Thus, the proliferation of the immune cells can be evaluated.

O. Immunological memory test

T cells from mice in control groups, single-neoantigen group, and the multiple-neoantigen group will be transplanted into naive wildtype C57BL/6 mice, respectively. These mice are immunologically compatible with the treatment groups. Each naive mouse will be injected with X577 cells subcutaneously and flow cytometry will be applied to measure the level of activated CD4 and CD8 T cells.

P. Measurement of the Intracellular Level of GSH

The level of GSH in tumor cells after treatment with Lapa nanoparticles and evaluated with GSH and GSSG assay kits (Beyotime Biotechnology, Jiangsu, China).

Q. Measurement of Extracellular Level of ROS in TME

The suspension of the tumor is tested with DCFDA - Cellular ROS Assay Kit. With the ROS level in TME, the extent of immunosuppression can be correlated with the ROS level in the TME.

R. Measurement of Tumor Size and Survival Rate

Tumor growth will be measured by a digital caliper on day 10 and volume will be calculated using the formula:

$V = \frac{1}{2} \times Length \times Width^2$, where length is the longest

dimension while the width is the shortest. The survival time of every mouse in every treatment is carefully recorded and drawn into graphs.

S. Statistical Analysis

All numerical data gathered from each group will be analyzed using the Student's T-test and ANOVA analysis to examine the statistical significance.

IV. Anticipated Results

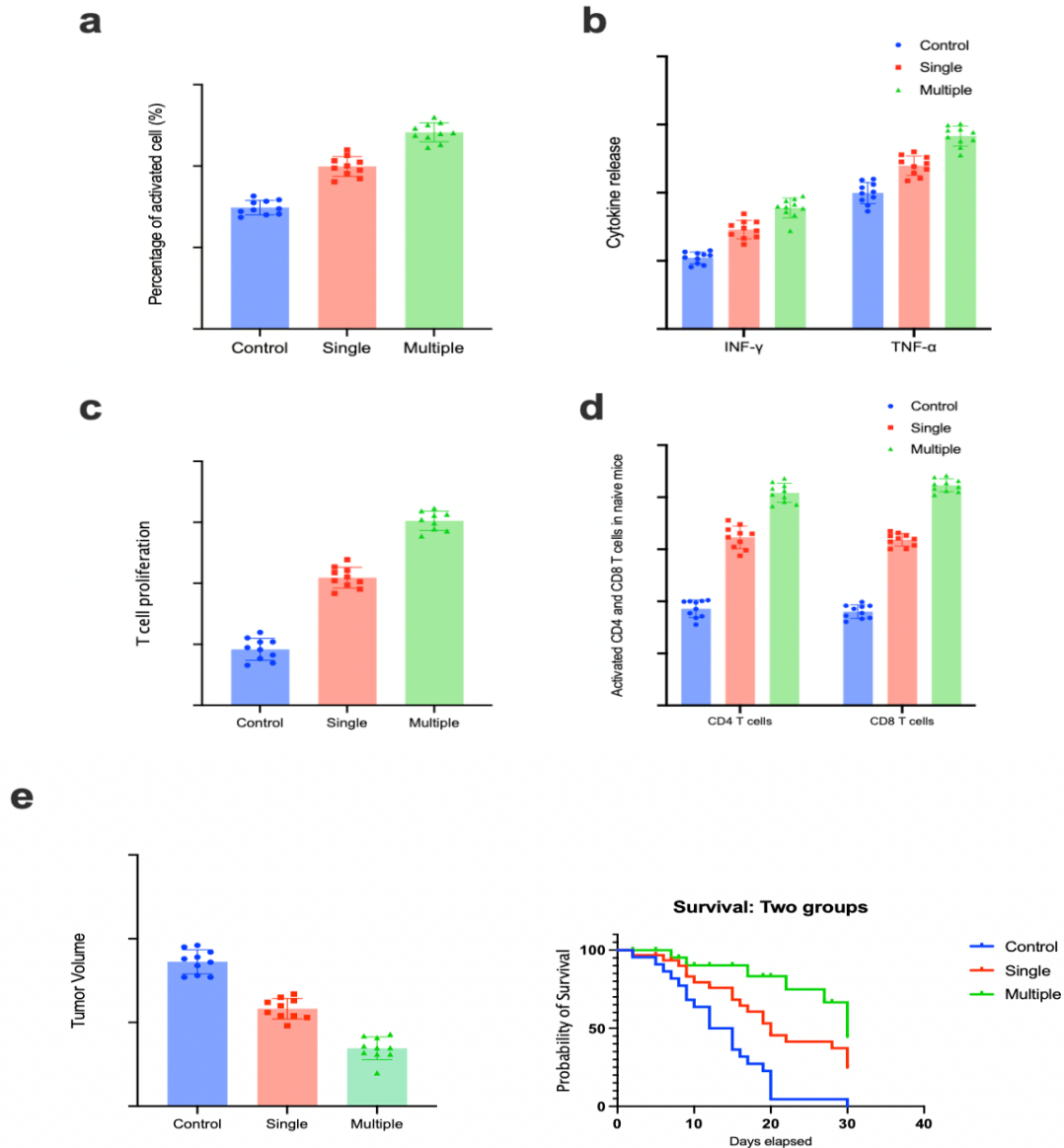## A. Efficacy of Multiple-Neoantigen Vaccine Compared with Single-Neoantigen Vaccine

Figure1. Predicted results of comparing multiple-neoantigen vaccines and single-neoantigen vaccines.

(a) More CD4 and CD8 T cells are activated by multiple-neoantigen vaccines.
(b) ELISA shows that multiple neoantigens triggers higher level of IFN-γ and TNF-α.
(c) T cells are more proliferative after injection of multiple-neoantigen vaccines .
(d) Higher immunological memory is induced by multiple-neoantigen vaccines .
(e) Multiple-neoantigen vaccines are also more effective on controlling tumor growth and increasing survival rate.

Subcutaneous injection of multiple-neoantigen vaccine induces robust T cell responses with higher levels of T cells, both CD4 and CD8 T cells, being activated and more IFN-γ and TNF-α being released. (Figures 1a and 1b) Results of the CCK-8 assay also confirm that vaccines with multiple neoantigens induce more vigorous T cell

proliferation than single neoantigen vaccines. (Figure 1c) The more vigorous T cell response might be ascribed to inhibition of tumor immune evasion[6]. Furthermore, naive mice receive T cells from different vaccine treatment groups and are then injected with tumor cells. Flow cytometry shows that T cells from multiple-neoantigen vaccine treated mice still have higher vitality in other naive immunologically compatible mice, indicating that

these T cells develop memory against this specific cancer and this anti-cancer response is T-cell dependent. (Figure 1d) Multiple-neoantigen vaccines are also more effective on tumor regression and exhibit higher survival rates, but the survival rates are still not desirable, possibly due to the immunosuppressive TME. (Figure 1e)

B. Change in TME and Immune Response in Response to Extracellular Scavenging of ROS
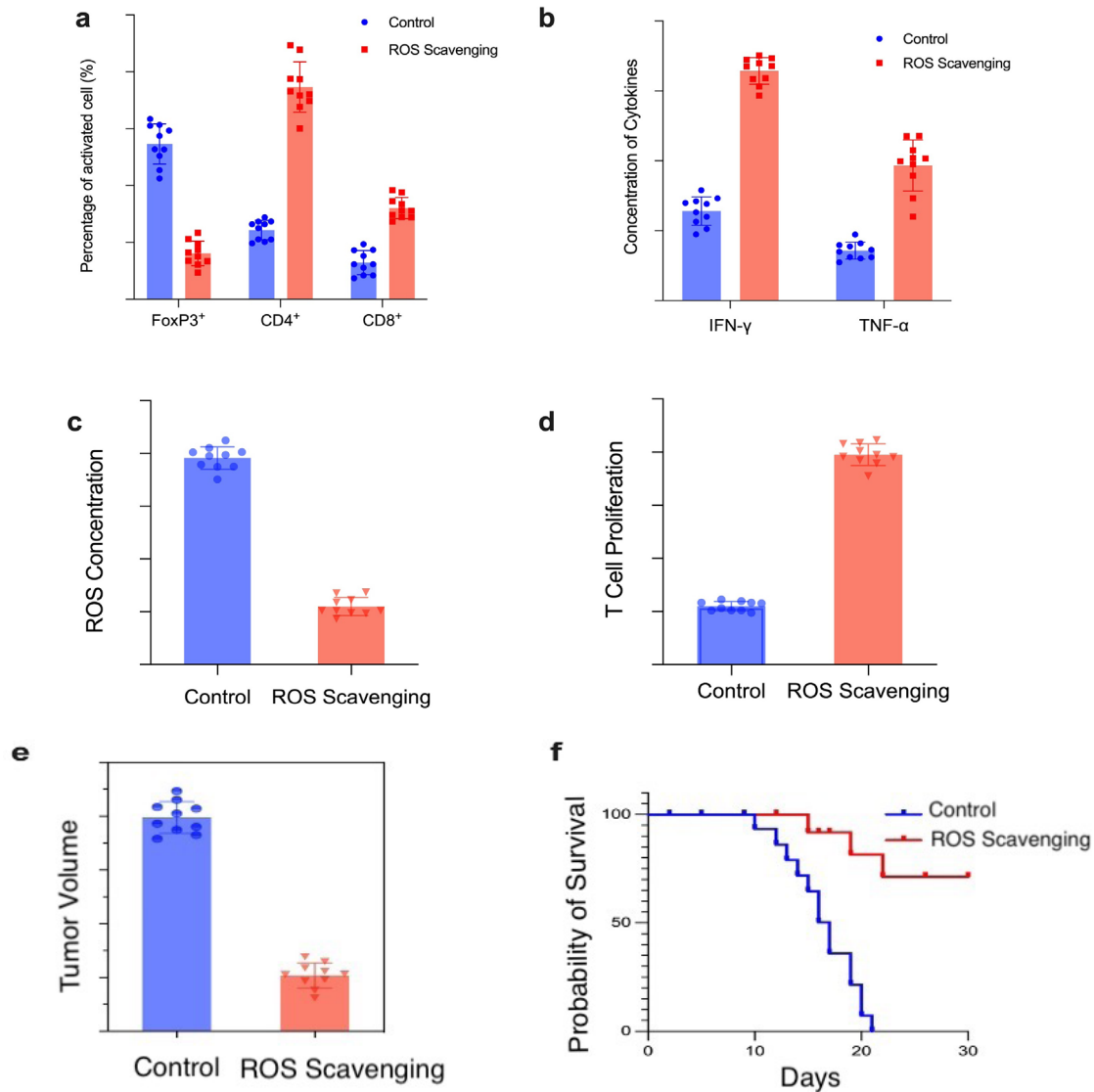

Figure 2: Predicted results of Extracellular ROS Scavenging Nanoparticles.

(a) Flow cytometry shows decreased regulatory T cells and increased CD4+ and CD8+ cells in TME which signifies a more robust immune response.

(b) ELISA shows increased IFN-γ and TNF-α production, and recruits more tumor-infiltrating lymphocytes.

(c) DCFDA - Cellular ROS Assay Kit shows decreased extracellular ROS concentration in TME, proving the efficacy of ROS Scavenging nanoparticles

(d) CCK8 Proliferation Test shows improved T cells vitality.

(e) Extracellular ROS Scavenging can effectively reduce tumor size.

(f) Mice receiving Extracellular ROS Scavenging show improved survival and prognosis.

T cell surface markers and the number of tumor-infiltrating lymphocytes are recorded through flow cytometry. Results confirm that nanoparticles create a more immunogenic TME by activating more CD4+ and CD8+ cells and suppressing regulatory T cell activation. (Figure 2a) ELISA shows a higher concentration of inflammation-inducing cytokines like IFN-γ and TNF-α in TME after the nanoparticles treatment.（Figure 2b) Decreased ROS concentration in TME also confirms

the efficacy and security of the nanoparticles. (Figure 2c) Activated T cells in TME show higher vitality in the CCK8 cell proliferation test, which will further enhance the duration and intensity of the immune response. (Figure 2d) Extracellular ROS Scavenging indeed can reduce tumor size and increase survival rate. (Figure 2e, 2f)

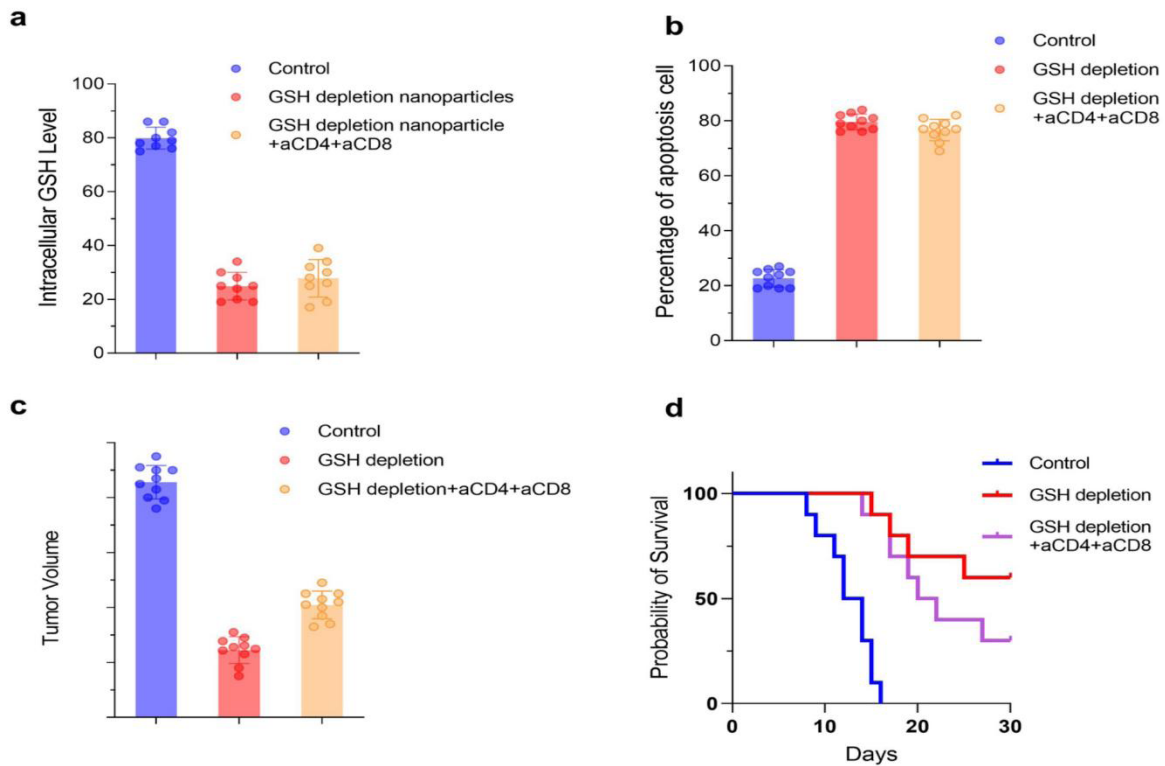*C. Increased Antitumor Effect in Response to Intracellular Depletion of GSH*

Figure 3：GSH depletion can lead to tumor apoptosis in a T cell-independent manner.
(a)The nanoparticles chosen can significantly decrease the intratumoral level of GSH in both WT mice and T cell deficient mice.
(b) The GSH depletion in tumor cells can lead to increased cell apoptosis, in a T cell-independent manner.
(c)(d) The GSH depletion can lead to decreased tumor volume and increased survival rate in both WT and T cell-deficient mice, but T cells still play an important role in attacking tumors.

Nanoparticles loaded with Lapa drugs are injected intravenously into WT mice and T cell-deficient mice. Intratumoral GSH levels can be measured by GSH and GSSG assay kits (Figure 3a). Then the percentage of apoptosis cells in tumor cell clusters are analyzed by flow cytometry using apoptosis-specific fluorescence markers. (Figure 3b) Then the tumor volume and survival rate of each group are measured, indicating that this type of nanoparticles indeed have anti-tumor efficacy, but the antitumoral efficacy is the best when T cells are present, since T cell deficient mice have larger tumors and lower

survival rate. (Figure 3c, 3d)

D. Efficacy of the Combinational Therapy

Combinational therapies significantly decrease tumor growth and increase the survival rate. MN vax: Multiple-neoantigen vaccine. (Figure 4a, 4b). Prior to the vaccine, nanoparticles are injected intravenously to relieve immunosuppressive TME and to cause tumor apoptosis. The subcutaneous injection of multiple-neoantigen vaccine then activates DCs and T cells to further boost immune response.
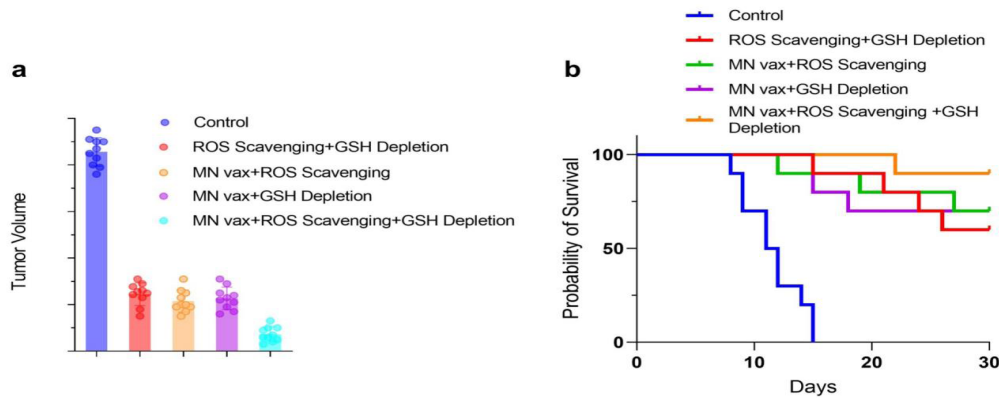
Figure 4：Combinational Therapy increases anti-tumor effects.

V. Potential Caveats

A. Multiple-neoantigen Vaccine

Although multiple-neoantigen peptide vaccines show a higher anti-cancer efficacy, there still exist several challenges. First of all, the production of this vaccine is complicated. Neoantigen generation requires the identification of nonsynonymous mutations and measurement of MHC affinity. Moreover, there are several complex criteria for the selection of neoantigens identified. Second, the application of the vaccine is highly restricted since those neoantigens are personalized and tumor-specific, so there does not exist a multiple-neoantigen peptide vaccine that works for everyone. The cost will also be very high.

B. Nanoparticles Scavenging Extracellular ROS

Even though those nanoparticles are highly specific to TME and very efficient in reducing ROS, there are still some defects. First, other immunosuppressive factors like Immune Checkpoint PD-L1 will still be up-regulated on tumor cells. The TME is still acidic and short of oxygen. Those factors may still inhibit immune response. So those nanoparticles must be combined with other treatments to achieve optimum effect. Some research shows that extracellular ROS scavenging archives the best effect when combined with PD-L1 treatment. (Weinberg et al., 2019) Second, the author uses ELISA to test cytokine concentrations in TME which might be too low to gain accurate readings due to considerations in budget. Intracellular staining will be a better option to more precisely reflect the change in cytokine secretion.

C. Nanoparticles Depleting Intracellular GSH

Although the nanoparticles deplete intratumoral GSH specifically through NAD(P)H:NQO1 catalysis and EPR (enhanced permeability and retention) effect, there is still

a possibility that these nanoparticles can act on normal cells. Normal cells maintain lower levels of intracellular ROS and antioxidants, but the depletion of antioxidant GSH can still cause apoptosis in normal cells. Also the excessive ROS accumulated in tumor cells may be released into tissue, which can lead to further tissue destruction.

D. Combinational Therapy

The novelty of this combinational treatment is accompanied by the uncertainty. It is rather unclear whether these independent therapeutic reagents will react with each other in vivo. Also the intervention on the activation of immune cells may lead to acute local inflammation and cytokine release syndrome.

VI. Conclusions

High intratumoral ROS directly promotes progression and metastasis of LUAD while high ROS in TME further disables T cells by increasing their oxidative stress. Frequent antigen depletion and antigen escape in LUAD also make treatment much less effective. Thus, multiple neoantigen vaccines combined with extracellular ROS scavenging and intracellular antioxidant GSH depletion can suppress tumor cell proliferation and promote the antigen-specific immune response. In this study, the healthy wild-type C57BL/6 mice are divided into 12 groups, each receiving different combinations of the three therapies or serving as control. One group receives aCD4 and aCD8 to test whether GSH-depletion-induced apoptosis is T-cell independent. Another group of naive mice receive T cells from the mice surviving tumor challenge prior to tumor implantation to test that the immune response triggered by the vaccine is T cell-dependent and produces immune memory. After orthotopic intrapulmonary implantation of X577 cells, the mice's tumor-infiltrating lymphocytes (TIL) are analyzed to show the intensity of immune response by

flow cytometry. The ratio of cytokines IFN-γ and TNF-α in the TME is also examined by ELISA to reveal the degree of immunogenicity in the TME. Some TILs are extracted and cultured in vitro to receive Cell Counting Kit 8 proliferation tests to test their vitality in TME. In intracellular antioxidant GSH depletion, the amount of tumor cells undergoing apoptosis is measured through flow cytometry to prove the efficacy of GSH depletion nanoparticles. To conclude, this therapy provides experimental designs and valuable insight for studying combinational therapies targeting ROS-related cellular activities and improving the efficacy of cancer vaccines. Most current studies focus on either ROS or cancer vaccine, rather than evaluating the effect of combinational therapy. This study fills the knowledge gap and presents a novel approach to alter TME, halt tumor growth, and improve the efficacy of cancer vaccines. Additionally, the experimental design in this study may apply to other combinational therapies, facilitating breakthroughs in the field of cancer research.

Acknowledgement

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