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Inducing Anti-Tumor Immune Responses in Lung Adenocarcinoma: High-Dose of Ascorbic Acid Combined with Arginine and Magnesium Sulfate

Yu H[1#], Wang R[1#], Gao Y[1#], Zhang J[1], Gao J[1], Lu F[1], Chen Z[1], Xia Y[1], Hou Y[1], Li L[1], Wang L[1], Moreira P[2,3], Chang L[1]* and Li W[1]**

[1]Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University, China

[2]International Healthcare Management Research and Development Centre, Shandong First Medical University, China

[3]Department of Biology, Medicine and Health, The University of Manchester, UK

[#]These authors contributed equally to this work

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Correspondence:
Paulo Moreira, International Healthcare Management Research and Development Centre, Shandong Provincial Qianfoshan Hospital, Shandong First Medical University, Jinan, Shandong 250014, China, E-mail: jpm2030@outlook.com
Li Chang, Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming, Yunnan 650118, China, E-mail: changli1981@126.com
Wenhui Li, Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming, Yunnan 650118, China, E-mail: 2998384689@qq.com

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Abstract

Evidence on the anti-cancer effect of high-dose Ascorbic Acid (AA) has been generated from several basic and clinical studies. AA can increase the level of Reactive Oxygen Species (ROS) through oxidative stress, modulate tumor immunity. However, a model using "continually intravenous pump of AA for 24 h" has not been widely reported. We treated 4 palliative patients using this approach combining the metabolic mechanisms of AA in the body and possible synergy mechanisms with arginine and magnesium ion. The results show significantly improved survival in two patients with advanced Lung Adenocarcinoma (LUAD) who received this combination therapy and no serious adverse events occurring. Therefore, we explored a high-dose AA combination with arginine and magnesium sulfate with exhibited synergistic anti-tumor effects in LUAD, which have significantly improved anti-cancer effects and enhance anti-tumor immunity in vitro and in vivo. This study provides a contribution to a debate on a new high-efficiency and low-toxicity combination therapy in future clinical practice, potentially improving the prognosis of patients with LUAD.

Graphical Abstract: Image 1.

Keywords: Ascorbic acid; Arginine; Magnesium sulfate; Lung adenocarcinoma; ROS; Anti-tumor immunity

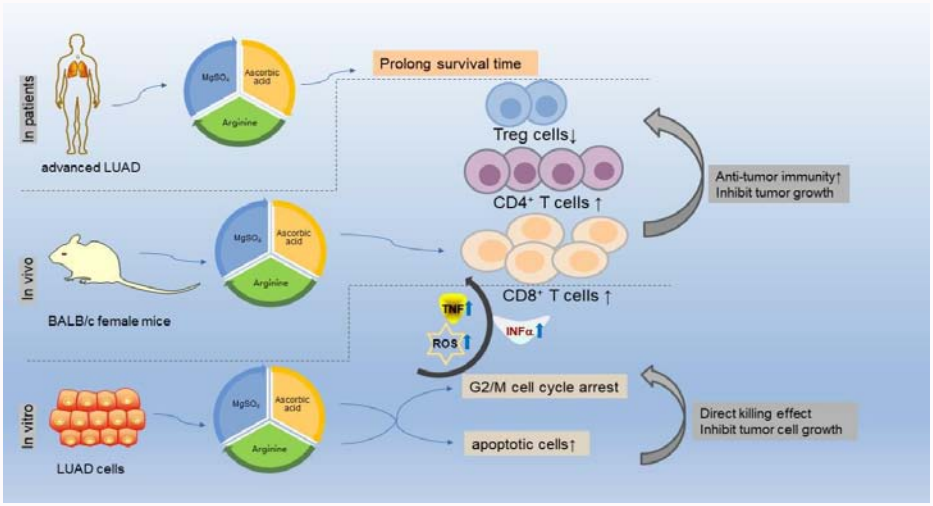

Image 1: Graphical abstract.

Highlights

- Of 4 dying patients who received "high dose Ascorbic Acid (AA)+arginine+magnesium sulfate" intravenous pump for 24 h, two patients with advanced LUAD had significantly improved their survival time.
- The combination of "high dose AA+arginine+magnesium sulfate" can significantly inhibit the growth of LUAD both *in vivo* and *in vitro*.
- "High doses AA+arginine+magnesium sulfate" combination can increase ROS level and raise cytokines associated with Interferon (IFN) signal.
- "High doses AA+arginine+magnesium sulfate" combination can significantly enhance antitumor immunity.

Introduction

In spite of a diversity of treatment options, the prognosis of advanced Lung Adenocarcinoma (LUAD) is still poor. In a global context in which new approaches to healthcare and cancer research are expected [1,2], also finding new treatments with high efficiency and low toxicity is a knotty problem to be solved. Within the past 10 years, there has been growing evidence that AA may become an effective anticancer drug when administered intravenously and in high doses rather than orally [3]. AA has been shown to trigger interferon responses to promote both the innate and adaptive immune responses by modulating T cells [4-6]. Importantly, it has been used in combination with some other therapeutic agents achieving synergistic therapeutic effects, such as chemotherapeutic drugs, DNA methyltransferase inhibitors, and even improved the efficacy of immune checkpoint therapy [7-9]. Some other studies suggest that AA with Arginine can exert their anti-tumor effects through redox, targeting HIF-1α, epigenetic regulation, and immune regulation [10] and that magnesium ions can significantly enhance the anticancer effect of AA and anti-tumor immunity [11,12]. However, the effect of combination with the three has not been widely reported. The model “continually intravenous pump of AA for 24 h” also has not been widely reported.

In clinical application, we treated 4 dying cancer patients who had received all treatments from the first through the third line with combination of AA with arginine and magnesium sulfate according to their family members’ strong requests. The combination regimen included arginine 7.5 g, AA 30 g to 100 g, magnesium sulfate 3.75 g, and potassium chloride 4 g continually intravenous drip for 24 h for 20 to 91 days. All patients showed to be safe. Two of them with LUAD were found to have significant improvement in survival. The clinical results displayed that this combination regimen was safe and probably effective in LUAD. In order to verify the potential antitumor activity and possible mechanism of this combination regimen in LUAD, this study designed *in vitro* and *in vivo* experiments for further study.

Results

High-dose vitamin C combined with arginine and magnesium sulfate seems safe and effective in patients with advanced cancer

Based on the above background international research, the authors envisioned whether the high-dose AA combination therapy can bring a glimmer of hope to end-stage cancer patients in clinical frontline work. Having informed the patients and obtained their informed consent and signed the relevant informed consent, four patients with advanced cancer were given arginine 7.5 g, AA 30 g, magnesium sulfate 3.75 g and potassium chloride 4 g intravenously for 24 h. Among them, one patient was advanced LUAD with multiple metastases of lung, liver, intracranial and bone. After multi-line treatment, the patient developed a large number of pleural effusions, chronic cardiac insufficiency, hypoproteinemia, moderate anemia and electrolyte disturbance. The estimated survival time was less than one month. Our joint program lasted for nearly 3 months, during which the general condition of the patients was satisfactory and symptoms such as pain and chest tightness were relieved. The chemotherapy and immunotherapy combined with this combination therapy were used. CT examination after treatment indicated that the disease was stable.

The other patient was a patient with advanced LUAD with intracranial and bone metastases. The patient received this combination treatment for 2 months and was discharged from the hospital in a state of consciousness disorder and coma. After discharge, the patient still survived for 4 months and regained consciousness. The highest dose of AA in both patients was 100 g/d.

Based on the improved survival of the previous two patients with end-stage malignancy, the authors used this combination therapy with other anti-tumor therapies in one patient with advanced breast cancer and one patient with advanced gastric cancer, but they did not seem to have the same efficacy as the first two patients.

Four patients aged 37 to 65 years who were diagnosed with stage IV malignancies at a Cancer Hospital from January to July 2022 received this treatment after first-line or above treatment, as detailed in Table S1.

Duration of medication ranged from 20 to 91 days. The dose of AA was adjusted according to the patient's condition and ranged from 20 g/d to 100 g/d. Simultaneous therapy during treatment included radiotherapy, chemotherapy and so on. Grade 3 AEs were recorded 9 events, including thrombocytopenia, fever, leucopenia, decreased hemoglobin and hypokalemia. During the treatment, the patients had other infections or were treated with other therapies, such as chemotherapy or peritoneal hyperthermic perfusion therapy. The physician evaluated that the Grade 3 adverse events were unrelated to this treatment. There were no Grade 4 or 5 adverse event occurred during treatment, detailed data are shown in Table 1 and Table S2.

This study suggests that high-dose AA combined with arginine and magnesium sulfate can be safe and effective in clinical application, and it can provide an alternative treatment for patients with advanced tumors, especially for LUAD.

However, to further verify the potential antitumor mechanism of this combination regimen, more experiments *in vivo* and *in vitro* have to be designed to explore and generate more evidence.

High-dose AA combined with arginine and magnesium inhibited cell growth *in vitro*

The effect of clinical preliminary medication has generated interest and inspiration. In order to further investigate the antitumor effect and potential mechanism of this combination on LUAD, this study firstly conducted a series of trials *in vitro*. The best concentration of Ascorbic Acid (AA), arginine and magnesium sulfate on lung cancer cells was explored by CCK8 experiment. We found that high concentration of AA had obvious killing effect on LUAD cells, with

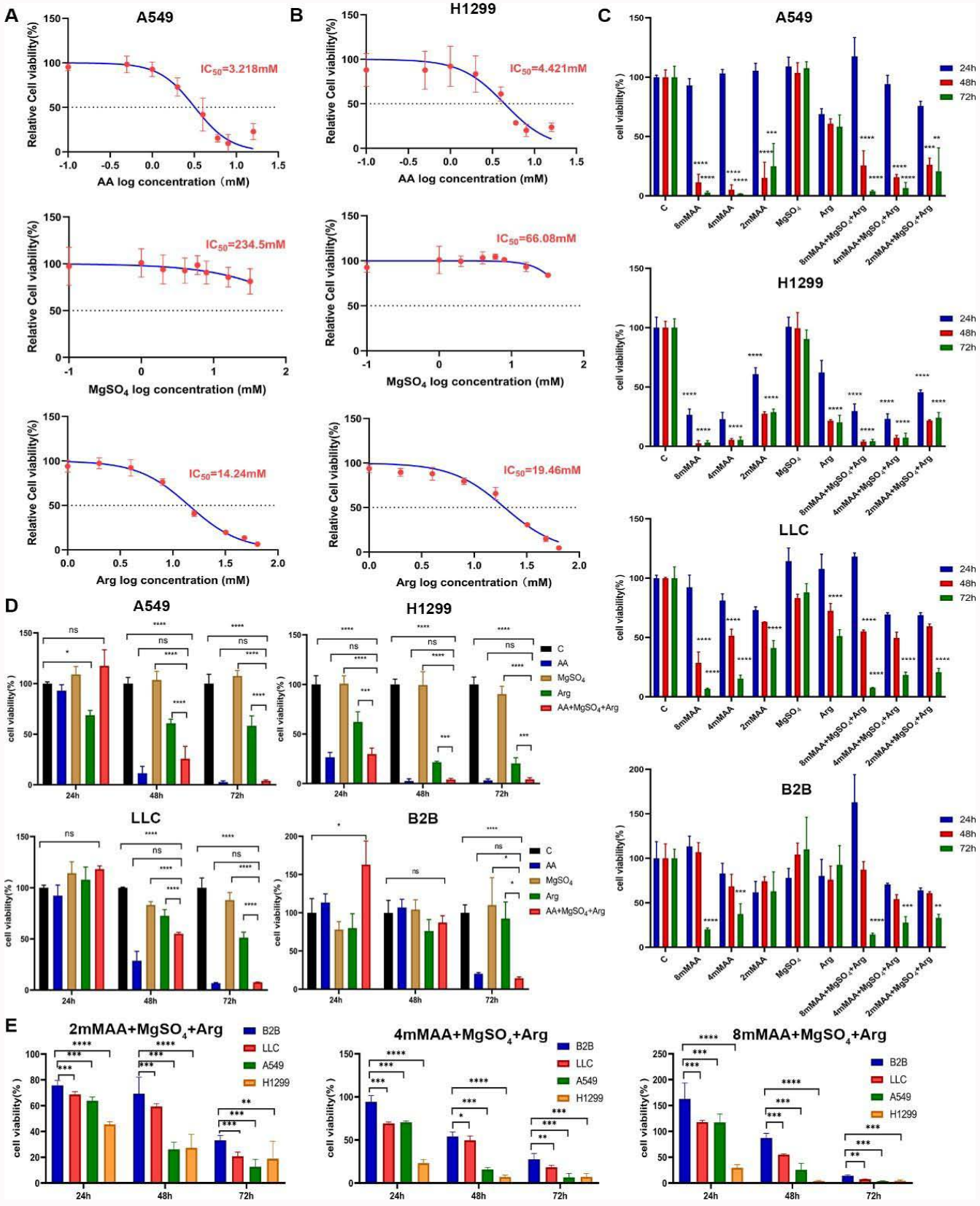

Figure 1: Combination of high dose AA, arginine and magnesium sulfate inhibit the growth of lung cancer cells.
(A & B) CCK8 assay was used to determine the changes of cell viability of A549 and H1299 cell lines treated with different concentrations of ascorbic acid (AA), arginine and magnesium sulfate. **(C)** Effects of different concentrations of AA, arginine, magnesium sulfate and the combination of them on the viability of A549 H1299 LLC B2B cells for 24 h, 48 h, 72 h. **(D)** Effects of 8 mM AA, arginine, magnesium sulfate and the combination of them on the viability of A549, H1299, LLC and B2B cells. **(E)** Comparison of the effects of combined use of different concentrations of AA, arginine and magnesium sulfate on the activity of B2B, LLC, A549 and H1299 cells. *: P<0.05; **: P<0.01; ***: P<0.005; ns: not significant

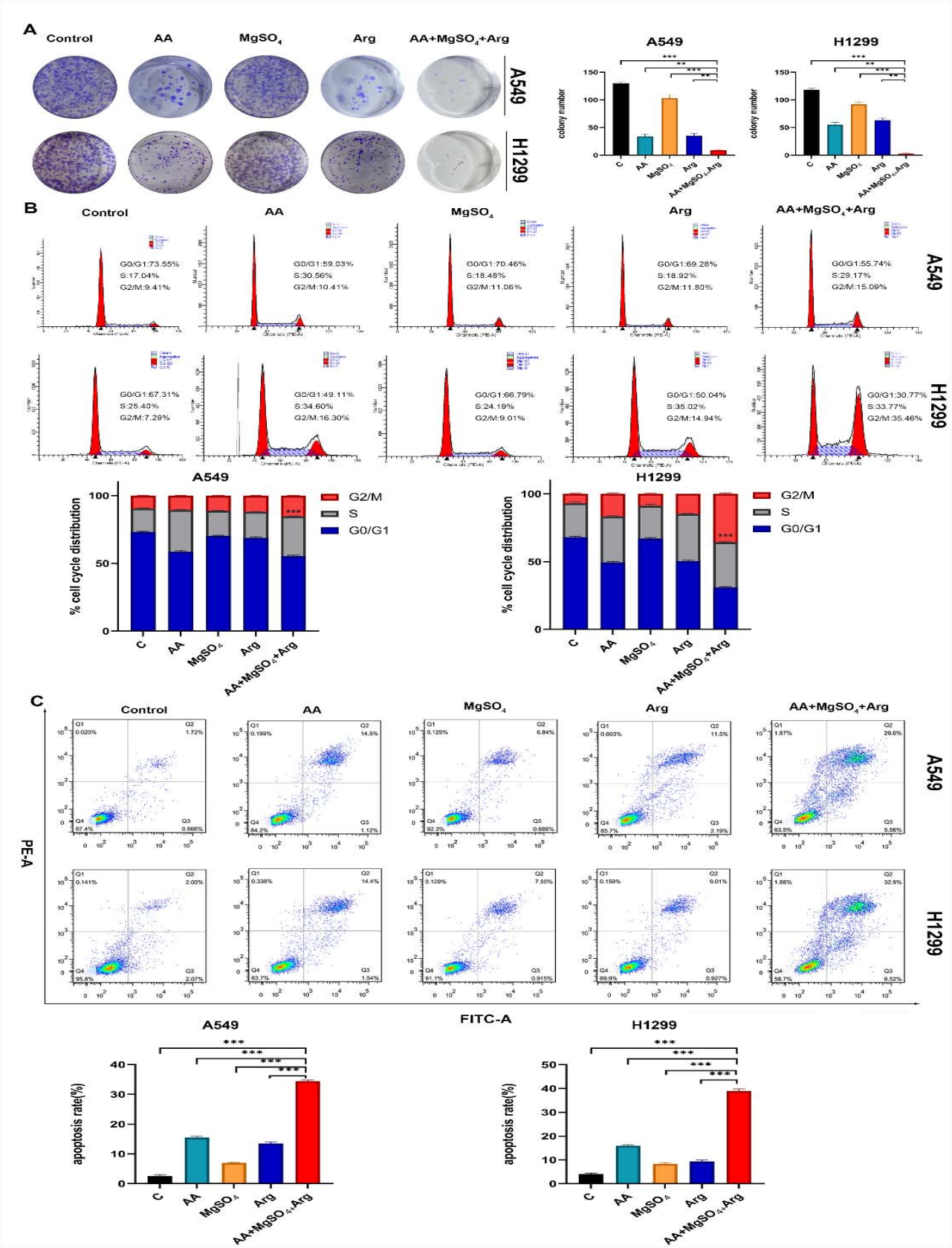

Figure 2: High-dose AA combined with arginine and magnesium affects cell proliferation, cell cycle and apoptosis.
(A) The clone formation ability of A549 H1299 cells in the AA, Arginine (Arg) and magnesium sulfate alone or in combination. (B) Changes of cell cycle after AA, arginine and magnesium sulfate alone or in combination treatment in A549 and H1299 cells by flow cytometry. (C) Detection of apoptosis of A549 and H1299 cells treated with AA, arginine and magnesium sulfate alone or in combination by flow cytometry.
**: P<0.01; ***: P<0.005; ns: not significant

the increase of AA concentration, the proliferation rate of LUAD cells decreased gradually. When the concentration of AA was 3.218 mM, the growth of A549 cells was inhibited by 50%. When the concentration of AA was 4.421 mM, the growth of H1299 cells was inhibited by 50%. A similar phenomenon was observed in arginine. When the concentration of arginine was 14.24 mM, it could inhibit the proliferation of A549 cells by 50%. When the concentration of arginine was 19.46 mM, it could inhibit the proliferation of H1299 cells by 50%. Magnesium sulfate had no obvious effect on cell proliferation (Figure 1A, 1B).

According to the above results, different LUAD cells and normal lung epithelial cells B2B were treated with varying doses of AA (2 mM, 4 mM and 8 mM), arginine (18 mM) and magnesium sulfate (1 mM) to observe the effects of the combination of the three and single drug on cell activity. After the treatment of AA, arginine, magnesium sulfate alone and the combination of three for 24 h, 48 h, 72 h. With the extension of the action time, the inhibitory effect on the cells was more obvious. We found that the combination of the three drugs and AA alone for 72 h and 48 h had more significant inhibitory effect on LUAD cells, and the difference was statistically significant (Figure 1C).

In addition, comparison of AA, arginine, magnesium sulfate and any combination of two drugs, the results are similar (Figure S1). For A549, H1299, LLC cells, the inhibitory effect of combined treatment group for 48 h and 72 h was more obvious than that of arginine and magnesium sulfate alone, but there was no significant difference compared with AA alone. Interestingly, the combination of three drugs showed a stronger anti-tumor effect on LUAD cells compared with normal lung epithelial cell B2B (Figure 1D, 1E).

Combination treatment with high-dose AA, arginine and magnesium effectively suppressed cell proliferation, blocked the cell cycle, and promoted cell apoptosis the antitumor effect of this combination was next evaluated in a variety of experiments *in vitro*. In order to further detect the effect of AA, arginine and magnesium sulfate alone or in combination, the colony formation experiment was carried out to explore the changes of cell proliferation after treatment with different drugs for 10 to 14 days, because the results of CCK8 experiment only detected the changes of cell activity after treatment with 24 h, 48 h and 72 h. The combination of high concentrations of AA, arginine, and magnesium sulfate could significantly inhibit cell proliferation. Compared with the control group, the number of cell clones formed by the combination of AA, arginine, and magnesium sulfate decreased significantly (Figure 2A). Next, cell apoptosis was analyzed by flow cytometry analysis, high-dose AA, arginine and magnesium combination treatment significantly increased the apoptosis rate of H1299 and A549 cell lines (Figure 2C). Then, we determined whether the enhanced cell toxicity observed in the combination treatments could result from changes in cell cycle. It was found that compared with AA, arginine, or magnesium sulfate monotherapy, the combined group could significantly block the cell cycle in G2/M phase (Figure 2B). These results indicated the potential of the combination treatment which could exhibit potent antitumor effects in LUAD cells.

High-dose AA combined with arginine and magnesium enhanced ROS-induced Immunogenic Cell Death (ICD) and upregulated cytokines related to Interferon (IFN) signaling *in vitro*

ICD constitutes a prominent pathway for activating anti-tumor immune, and ROS production are vital components of intracellular pathways regulating ICD. Through the detection of ROS, it was found that the production of ROS in the combined group was higher than AA, arginine, or magnesium sulfate monotherapy and the difference was statistically significant ($p<0.01$) (Figure 3A, 3B). ICD constitutes a prominent pathway for activating the immune system against cancer, the secretion of cytokines associated with T cell activation or response, including IFN-γ and TNF-α were detected by Enzyme-Linked Immunosorbent Assay (ELISA). The results showed that the level of IFN-γ and TNF-α were significantly increased in the AA, arginine, and magnesium treatment group compared with the monotherapy group (Figure 3C, 3D). In conclusion, the combination therapy of three could promote ROS production leading to immunogenic tumor cell death, ultimately promoting the activation of cytokine IFN-γ and TNF-α. This result provides a theoretical basis in cell lines for further exploring the anti-tumor mechanism of the combination of the three drugs.

High-dose AA combined with arginine and magnesium therapy effectively suppressed tumor growth and improve anti-tumor immunity *in vivo*

To investigate the underlying effect of the combination of the three drugs *in vivo*, we used 6 to 8-week-old BALB/c female mice to establish a subcutaneous transplanted tumor model. These mice underwent the combination therapy according to the treatment scheme shown in Figure 4A. Compared with the control group and single drug group, a remarkable tumor suppression was observed in tumor-bearing mice administered a combination therapy of 1 g/kg and 4 g/kg AA, arginine, and magnesium (Figure 4B, 4C). Then, the results showed that compared with the control group and single drug group, the tumor volume of mice in the 1 g/kg AA or 4 g/kg AA plus arginine, magnesium sulfate group decreased significantly, there was no significant change in tumor weight (Figure 4D, 4E), and there was no significant difference in body weight among the seven groups (Figure 4F). It is proved that the combination of high concentrations of AA, arginine, and magnesium sulfate plays a strong anti-tumor effect *in vivo*. The success of tumorigenesis was further confirmed by immunohistochemistry of tumor tissue at the same time (Figure S2). To further explore the anti-tumor mechanism of the drug combination *in vivo*, we collected the peripheral blood of mice to analyze the T cell profiles by flow cytometry. The 1 g/kg AA combination therapy group and 4 g/kg AA combination therapy group significantly increased the number of CD3+ T cells in peripheral blood compared with the control group and single drug group (Figure 4G, 4I). T cell subset analysis revealed that the percentages of CD8+ T cells significantly increased after 1 g/kg AA combination therapy and 4 g/kg AA combination therapy compared with control group (Figure 4H, 4J). Taken together, these results suggested that 1 g/kg AA, arginine, and magnesium combination therapy could exhibit potent antitumor effects in mice. 1 g/kg AA and 4 g/kg AA combined group can increase CD8+ T cells, and enhance anti-tumor immunity in mice, which provides a theoretical basis for revealing the anti-tumor mechanism of high concentration of AA, arginine, and magnesium sulfate.

Combination therapy increased the number of CD8+ T cells and significantly reversed the immunosuppressive tumor microenvironment

It is widely recognized that T cells play a key role in antitumor immunity. We found that 1 g/kg AA combined group and 4 g/kg AA combined group could significantly increase the level of CD8+

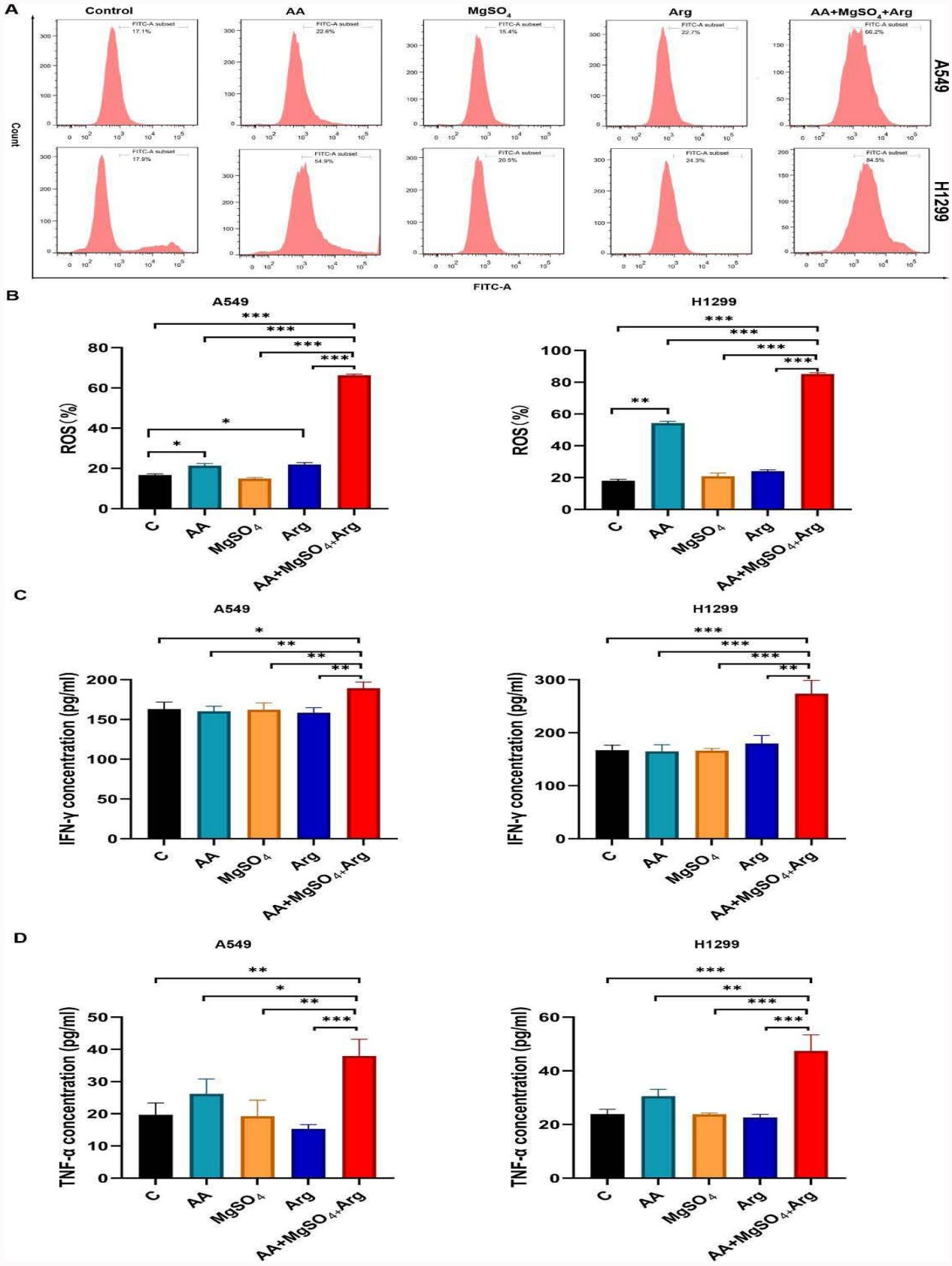

Figure 3: The combination of high-dose AA and arginine and magnesium sulfate increase the proportion of ROS, IFN-γ and TNF-α.
(A & B) Flow detection of reactive oxygen species in A549 and H1299 cells treated with AA, arginine and magnesium sulfate alone and combined group of three drugs. (C) Detection of IFN-γ changes in A549 and H1299 cells after AA, arginine and magnesium sulfate alone and combined group of three drugs treatment by ELISA. (D) TNF-α changes after AA, arginine and magnesium sulfate alone and combined group of three drugs treatment in A549 and H1299 cells were detected by ELISA. Data are shown as means ± SEM. *: P<0.05; **: P<0.01; ***: P<0.005; ns: not significant

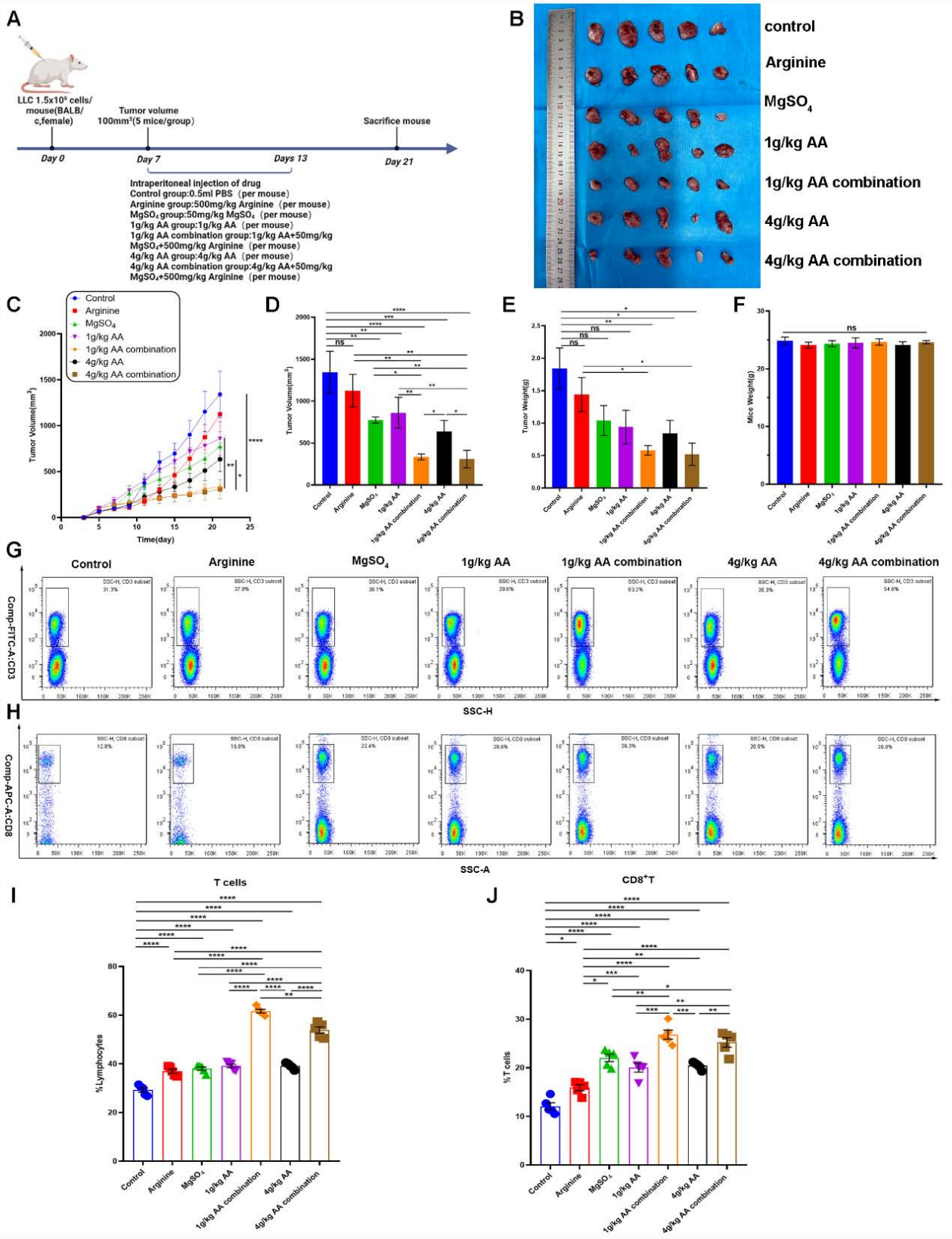

Figure 4: Effects of AA combined with arginine and magnesium sulfate on LUAD xenotransplantation model and anti-tumor immunity.
(A) Schematic of drug administration for animal experiments. (B and C) Gross view and growth curve of tumors in control group, single drug group and combination group. (D-F) Statistical diagram of tumor weight, tumor volume, and mouse weight in control group, single drug group and combination group. (G) Results of flow cytometry detection of T cells in peripheral blood of mice. (H) Peripheral blood CD8+ T cells of control group and combination group were detected by flow cytometry. (I and J) Statistical chart of T cells and CD8+ T cells in peripheral blood. Data are shown as means ± SEM. *: P<0.05; **: P<0.01; ***: P<0.005; ns: not significant

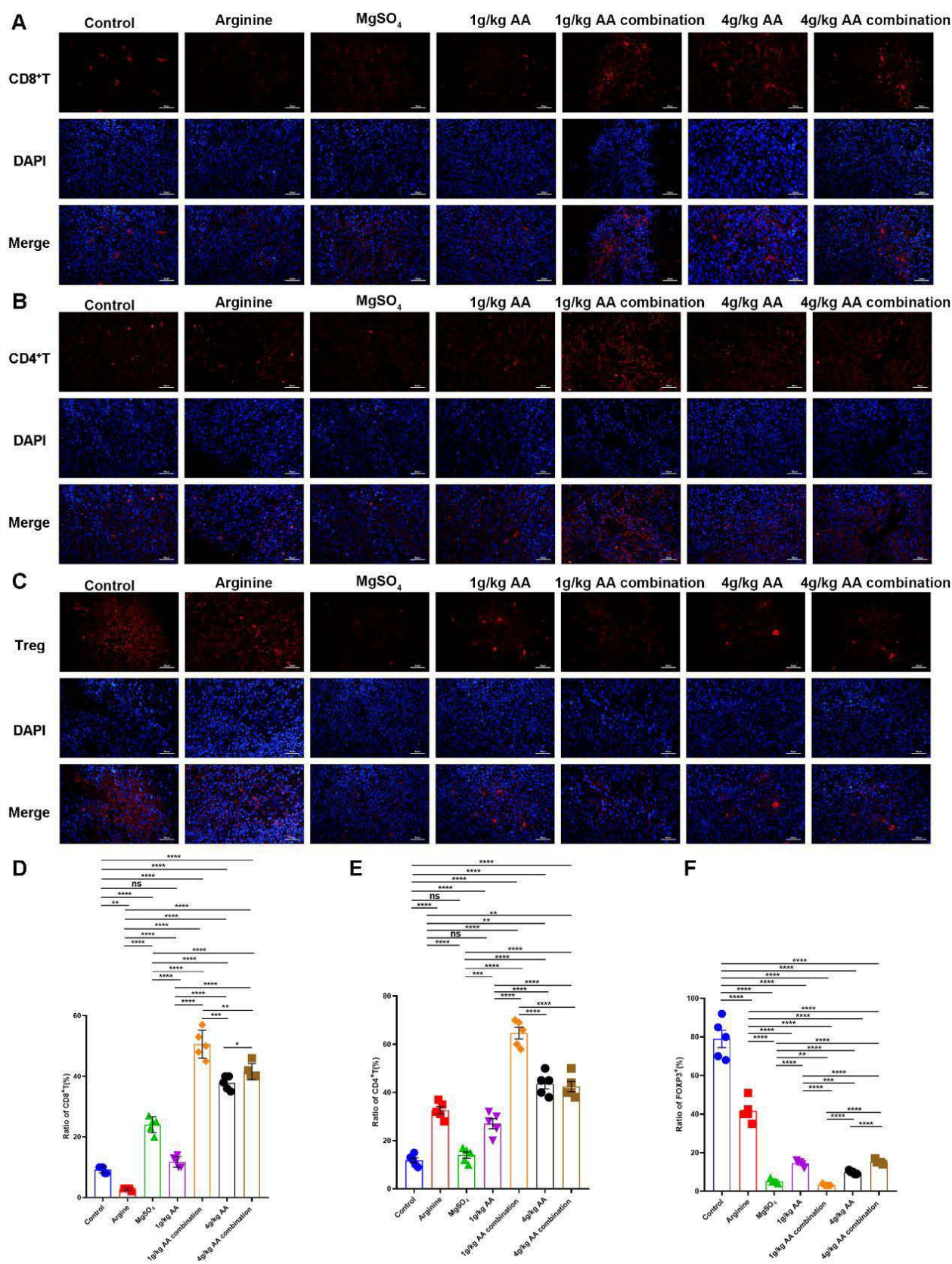

Figure 5: High-dose AA combined with arginine and magnesium increase immune response.
(A) Immunofluorescence detection of CD8+ T cells in tumor tissue of mice. (B) Immunofluorescence detection of CD8+ T cells in tumor tissue of mice. (C) Immunofluorescence detection of Treg cells in tumor tissue of mice. (D) Statistical analysis of CD8+ T cells in tumor tissues. (E) Statistical analysis of CD4+ T cells in tumor tissues. (F) Statistical analysis of Treg cells in tumor tissues. Data are shown as mean ± SD; ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001, ****: P<0.0001.

Table 1: Drug use, adverse events, and OS of patients.

Patients	Administration time (d)	AA dose (g/kg)	AA dose (g/d)	Simultaneous anti-tumor therapy	Adverse events	OS(d)
1	20	0.42	30	immunotherapy, chemotherapy	none	30
2	91	0.33-1.67	20-100	immunotherapy, radiotherapy	Thrombocytopenia (Grade 3)	114
3	22	0.73-1.46	30-60	chemotherapy	Fever (Grade 3); Thrombocytopenia (Grade 3), Hemoglobin decrease (Grade 3), Leucopenia (Grade 3), Hypokalemia (Grade 3)	27
4	67	0.63-2.21	30-100	chemotherapy, vascular targeted therapy, immunotherapy	Fever (Grade 3), Hypokalemia (Grade 3)	230

T cells in peripheral blood and enhance anti-tumor immunity in mice (Figure 4H); however, the changes of local tumor tissue and immune microenvironment were not known, so we further detected CD8+ T cells, CD4+ T cells and Treg cells in tumor tissue. Results of immunofluorescence staining revealed that 1 g/kg AA combination therapy increased the numbers of CD8+ T cells and CD4+ T cells in the tumor tissues (Figure 5A, 5B). 4 g/kg AA combination therapy increased the numbers of CD8+ T cells in the tumor tissues, but there was no significant difference in the increase of CD4+ T cells in tumor tissue compared with the group treated with 4 g/kg AA alone (Figure 5D, 5E). Then, our study mainly explored the effects of combination therapy on immunosuppressive cells mostly about regulator T cells (Tregs). The 1 g/kg AA combination therapy group also significantly reduced the proportion of Tregs (FOXP3+) (Figure 5C, 5F). Although there was no significant difference in the effect of 1 g/kg AA combined group and 4 g/kg AA combined group on increasing CD8+ T cells in peripheral blood of mice, the effect of 1 g/kg AA combined group was better in improving tumor microenvironment, increasing CD8+ T and CD4+ T cells and reducing Tregs in tumor tissue. Studies have suggested that the combination of 1g/kg AA combined group can significantly improve the anti-tumor immunity and improve the tumor microenvironment both in the whole body and locally, which provides a theoretical basis for revealing the anti-tumor mechanism of the combination.

Discussion

LUAD accounts for about 55% of lung cancer. For patients with advanced LUAD, the main treatment methods are radiotherapy, chemotherapy, immunotherapy, targeted therapy, and so on [13,14]. But the prognosis of advanced LUAD is still poor and cancer imposes an economic burden on the human population. There have been continued efforts aiming to investigate novel therapeutic strategies to improve clinical outcomes and a quasi-consensus suggests that combination therapy may prove to be more promising than monotherapy.

In this study, a new combination of "high-dose AA combined with arginine and magnesium sulfate" is proposed which to our knowledge may be amongst the first experiments of the kind in the World.

As reported, this study observed 4 patients with advanced tumors who received the combination treatment here proposed. It has been reported in previous literature that high dose vitamin C is safe for patients with pancreatic cancer and shows the possibility of prolonging the survival time of patients [15]. In patients with ovarian cancer, high-dose ascorbic acid combined with traditional chemotherapy drugs carboplatin and paclitaxel enhanced anticancer effect and reduced chemotherapy- related toxicity [16]. High dose vitamin C plus chemotherapy may be beneficial to metastatic colorectal cancer patients with RAS mutation [17].

Most of the previous studies used intravenous administration. However, in this study, continuous intravenous pump was used, which is a novel approach to drug administration. The concentration of AA in the human body can maintain a high level of dynamic balance. The administration time in this study depended on the tolerance of the patients. The treatment time of the patients in the group varied from 21 to 91 days, and 2 of them took the drug for more than 2 months. In previous studies, the medication time was mostly 4 weeks, but no study lasted for 3 months as was the case in our study, which provided a set of new evidence on the debate about determining the time of drug use in the future. Our clinical practice has proved that long-term infusion of 30 g to 60 g of AA seems to be tolerable, which can provide a new reference basis for the selection of medication and dose for further clinical trials and alternative schemes for patients with advanced cancer.

There were 9 events of grade 3 adverse events recorded and no Grade 4 or 5 adverse events occurred during treatment. The physicians evaluated that the Grade 3 adverse events were unrelated to this treatment. Intravenous administration potentially increases the risk of urinary oxalate crystallization. But in this study, only one patient showed an increase in the number of crystals during a routine urine test, which did not cause related functional damage. Grade 3 thrombocytopenia was observed in two patients, one received AA 100 g/d, another occurred two days after chemotherapy. Our clinical practice has suggested that long-term infusion of 30 g to 60 g AA is safe and tolerable. This study used continuous intravenous pumping. In previous studies related to intravenous injection of high-dose AA, intravenous infusion was used for 3 or 4 times a week. No study to our knowledge has used continuous intravenous 24 h pumping, which suggests a potential new method. The pharmacokinetics of intravenously injected AA showed that from 8 h to 24 h, the concentration of the drug in blood, tissue fluid and cells remained in dynamic balance, similar to recent evidence [3,18]. So, this study chose continuous intravenous pumping for 24 h, which can maintain a high level of AA concentration in the human body. In previous studies, the duration of medication was mostly 4 weeks. But in this study the patients received treatment ranged from 0 to 3 months, 2 patients received for more than 2 months. Based on this unique dose frequency, this study provides a potential new option for the choice of medication time in the future.

In order to further investigate the antitumor effect and potential mechanism of this combination on LUAD, this study also conducted *in vivo* and *in vitro* trials. Although the results of CCK8 tests showed that the effects of AA, arginine and magnesium sulfate were not significantly different from AA alone *in vitro*, considering that the longest observation of CCK8 test was 72 h, it could not well detect the long-term effect of the drug, and the results of clone formation test which showed that the combined drug group was better than the

single drug group.

Experiments *in vitro* showed that high-dose AA combined with arginine and magnesium sulfate could block the cell G2/M phase, promote cell apoptosis, trigger the aggregation of ROS to promote Immunogenic tumor Cell Death (ICD) and up-regulate cytokines related to interferon signal to play a synergistic anti-tumor effect. *In vivo*, not only the proportion of CD8+ T cells in peripheral blood increased, but also the proportion of CD8+ T cells and CD4+ T cells in tumor tissue increased significantly, while the proportion of Treg cells decreased, indicating that this combination can promote local and systemic immune response and produce strong anti-tumor effect *in vivo*.

It has been reported in recent literature that magnesium ion is very important for the activation of CD8+ T cells [19]. L-arginine can improve anti-tumor immunity, and high-dose AA can enhance anti-tumor immunity [8,20,21]. Most of the doses of AA reported in previous literature are 4 g/kg. This study, however, suggests that the combination of the three may show a strong anti-tumor effect *in vivo*, and the effect of the 1 g/kg AA combined group was arguably better than that of 4 g/kg AA combined group. When AA was 5 g/kg, the mice died, indicating that the dose of AA should be less than 5 g/kg in animal experiments.

AA was first found to be named L-ascorbic acid because it can treat anti-scurvy. Due to the lack of the L-gluconolactone oxidase gene in the human body, it cannot synthesize AA. AA exerted functions under different conditions. AA can kill cancer cells by disrupting Fe^{2+} metabolism and generating hydrogen peroxide via prooxidant effects [9,16]. High-dose AA can be used as an oxidant to exert anticancer activity, exert cofactor activity, regulate collagen formation, participate in epigenetic regulation, and target ischemia inducible factor HIF-1α signal transduction to participate in immune regulation, as recent evidence suggests [18,22-24]. AA restrains the growth of aggressive tumors by stimulating the accretion of substantial amounts of ROS both *in vitro* and *in vivo* [25]. In thyroid cancer, AA plays a role through ROS-dependent inhibition of MAPK/ERK and PI3K/AKT pathways [25].

In addition, AA can also exert anticancer effects by acting on JAK/STAT and NF-κB pathway [26,27]. Magnesium sulfate is a magnesium supplement, which is commonly used in anticonvulsants, treatment of preeclampsia and eclampsia, preterm delivery, pregnancy-induced hypertension, constipation, and so on. Abnormal magnesium uptake is associated with a variety of diseases, including bacterial infections and tumors. Magnesium is the activator of SVCT-2. SVCT-2 plays an important role in AA uptake and activation of the JAK/STAT pathway [28]. Magnesium supplementation can inhibit the growth-promoting effect of low-dose AA on tumors and enhance the anticancer effect of AA [11]. Arginine can effectively increase the therapeutic effect of radiotherapy [29]. In prostate cancer, arginine activates the mTOR pathway, ultimately leading to up- regulation of nuclear coding Oxidative Phosphorylation (OXPHOS) genes [30]. In addition, it has been previously reported that arginine can play its role through the PI3K/AKT and RAS/ERK, JAK/STAT, NF-κB pathways [31-33]. Meanwhile, arginine plays an important role in promoting T cell function and anti-tumor immunity [34].

This present study has additionally generated evidence suggesting effects increased ROS production after AA combined with arginine and magnesium sulfate treatment. This novel combination strategy can aim to overcome the limitations of both drugs and maximize therapeutic benefits. In this combination strategy, AA could directly suppress tumor growth firstly. Then it remarkably seems to potentially further enhance the ICD effects and immune activation, which were considered as dominant roles of AA in this strategy. Hence, it also affected the number and activation of T cells in tumor tissues.

The results in this study, suggest that the combination of AA, arginine and magnesium sulfate holds the potential to increase the secretion of IFN-γ and TNF-α, and IFN-γ and TNF-α could activate T cells, promote the differentiation of CD8+ T cells, and enhance the anti-tumor immunity.

It has been reported in recent evidence that IFN-γ and TNF-α can play a synergistic role through the JAK/STAT and NF-κB pathway [35-37]. So, the combination of AA, arginine and magnesium sulfate can play a synergistic anti-cancer role may *via* affecting the PI3K/AKT, JAK/STAT or NF-κB signaling pathways to regulate anti-tumor activities and promote anti-tumor immunity, although further experimental verification is needed.

Several studies have shown that drug combination therapy has a wider application prospect in clinical practice. Zhang et al. proposed the combined treatment of "radiotherapy + PD-1 inhibitor + granulocyte giant cell stimulator", that is, " PRaG ". The toxicity of the PRaG regimen was manageable, and the triple therapy regimen benefited patients with advanced refractory tumors [38].

The combination of high-dose AA with deep hyperthermia can significantly prolong the survival period of tumor patients and improve their quality of life [39]. Related studies showed that the mixture (lysine, proline, arginine, AA, and green tea extract) can inhibit tumor growth and metastasis [40]. In the mouse model, the combination of ascorbic acid and chemotherapy drugs carboplatin and paclitaxel can synergistically inhibit ovarian cancer and reduce the chemotherapy- related toxicity of ovarian cancer patients [16]. In addition, AA may enhance immunotherapy, and combining the two is arguably a promising treatment [24]. This evidence suggest that that high-dose AA combined with other treatments is a promising treatment [22]. Considering that the implementation of high-dose AA may be a breakthrough in treating cancer patients with poor prognosis and few available treatment schemes, conducting further clinical experiments on this promising non-toxic cancer treatment mode is not only necessary, but also very urgent.

This study observed that high-dose AA combined with arginine and magnesium sulfate promoted good results in two patients with advanced LUAD who had failed first-line treatment, reached Stable Disease (SD) and extended the expected survival time of the patients at the same time. Meanwhile, the combination was demonstrated to be safe and well tolerated, and further the study generates evidence to argue that high- dose AA combined with arginine and magnesium sulfate may be a strong anti-tumor effect both *in vivo* and *in vitro*. It can directly kill tumor cells and significantly enhance anti-tumor immunity to play a synergistic role in tumor inhibition. More importantly, this combination is economically feasible and can be used in clinical practice, with high drug accessibility, broad application prospects and translational clinical value.

Star Methods

Experimental model and subject details

Cell lines and animals: Human adenocarcinoma A549 cells,

H1299 cells, normal lung epithelial B2B cells, and mouse LUAD LLC cells were purchased from the American Model Culture Bank (ATCC). Among them, A549 cells and H1299 cells were cultured in 37°C, 10% fetal bovine serum RPMI1640 medium (Dalian, Mellon). B2B cells and LLC cells were cultured in DMEM medium (Dalian, Mellon) containing 37°C and 10% fetal bovine serum. The animal experiment program was approved by the Ethics Committee. 6 to 8-week-old male BALB/c mice were purchased from the Institute of Zoology, Chinese Academy of Sciences, and the feeding conditions were suitable.

Build 6 to 8 weeks BALB/c female mice with subcutaneous tumors in the middle of the back. The dose of AA is 1 g/kg and 4 g/kg, magnesium sulfate is reported in previous experiments with 100 mg/kg [41], arginine is used with 500 mg [42], our experiment is a combination of three drugs. Central inhibition occurs when the concentration of magnesium sulfate is too high in the abdominal cavity. So, the magnesium sulfate dose is halved, the final dose of magnesium sulfate is 50 mg/kg, the dose of arginine is 500 mg/kg. When the volume of subcutaneously transplanted tumor reaches 100 mm^3, divide the mice into three groups at random and start intraperitoneal injection treatment, as follows: (i) control group, with normal saline for 7 consecutive days; (ii) 1 g/kg AA combined with 500 mg/kg arginine combined with 50 mg/kg magnesium sulfate, (iii) 4 g/kg AA combined with 500 mg/kg arginine combined with 50 mg/kg magnesium sulfate were administered for 7 consecutive days. After 21 days of treatment, all mice were euthanized, and pathological staining was performed and determined the weight and volume of the tumor.

CCK8 assay: Cells (4000-6000 cells per well) were inoculated in 96-well plates. After 24 h of culture, the cells were treated with different concentrations of AA, arginine, and magnesium sulfate for a specified time. Then Cell Counting Kit-8 was performed to evaluate the effect of the drug on cell viability and the IC50 value was calculated. The cells were treated with AA, arginine, and magnesium sulfate alone, or 2 drugs and 3 drugs for 24, 48, 72 h, and the cell proliferation rate was calculated.

Colony formation assay: Cells (800/well) were inoculated in 60 mm dishes, treated with AA, arginine, and magnesium sulfate for 48 h, and then cultured in RPMI-1640 or DMEM medium containing 10% fetal bovine serum for 8 to 12 days. The colonies were fixed with 4% paraformaldehyde, washed with PBS, and stained with crystal violet. Each assay was performed in triplicate.

Detection of Reactive Oxygen Species (ROS): The cells were seeded in 6-well plates. After 24 h of culture, cells were washed with PBS, and cultured in RPMI1640 containing 8 mM AA, 16 mM arginine, and 1 mM magnesium sulfate for 48 h, then washed and suspended in serum-free medium containing 10 uM DCFH-DA (Beyotime, S0033S). Incubate in dark at 37°C for 60 min. Next, the cells were washed and analyzed by flow cytometry.

Cell apoptosis assay: The cells were treated with 8 mM AA, 16 mM arginine, and 1 mM magnesium sulfate for 48 h, and then stained with an annexin V-FITC/PI apoptosis detection kit (Procell, Wuhan). The apoptosis rate was detected by flow cytometry. Each experiment was carried out in triplicate.

Cell cycle assay: The cells were treated with 8 mM AA, 16 mM arginine, and 1 mM magnesium sulfate for 48 h, then fixed overnight in a refrigerator with 75% ethanol at 4°C, washed off the fixative with PBS the next day, and stained with DNA Content Quantitation Assay (Cell Cycle) (solar, Beijing). Detected by flow cytometry. Each experiment was conducted three times.

ELISA: The supernatant of the cells treated with different drugs for 48 h was used to determine the corresponding cytokines with TNF-α ELISA kit (Boster, Wuhan) and IFN-γ ELISA kit (Jingmei, Jiangsu). Samples and standard samples were added and reacted at 37°C for 90 min. Biotin labeled antibody was added and reacted at 37°C for 60 min. 1x washing buffer was washed for 3 times. Reaction at ABC, 37°C for 30 min. 1x washing buffer was washed for 5 times. Reaction at TMB 37°C for 15 min to 20 min. The terminating solution was added and O.D. Was determined by enzyme labeling instrument in 450 nm.

Hematoxylin-eosin staining: After the mice were killed, the tumor and muscle tissues of the mice were taken for hematoxylin-eosin staining, and then observed under the microscope and photographed.

Flow cytometry detects CD3+ T cells and CD8+ T cells: Collect the peripheral blood of mice and add 0.5% heparin sodium. Add the required antibodies of CD3, CD4, and CD8a (4A biotech) respectively. Incubate at room temperature and away from light for 20 min. Add red blood cell lysate. After cleaning, perform flow cytometry detection.

Immunofluorescence staining: The tumor tissue was taken for immunofluorescence staining of CD8+ T cells, CD4+ T cells and Treg cells. The tumor tissue was dewaxed to water on paraffin sections. After antigen repair, the tumor tissue was circled and sealed with serum: The corresponding first antibody (Servicebio, Wuhan) was added, then the second anti-CY3 red light reagent was added, and the nucleus was re-stained with DAPI. The sections were observed under fluorescence microscope and the images were collected.

Quantification and statistical analysis

All of the statistical analyses were performed using Prism software, version 8 (GraphPad Software). All data are expressed as mean ± Standard Error of Mean (SEM) from at least three independent experiments ($n \geq 3$). Student's t test (comparisons between two groups), one-way ANOVA with Tukey post hoc (comparisons of three or more groups with one independent variable) were used as indicated in the legends. $p<0.05$ was considered statistically significant (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, ****: $P<0.0001$).

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