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ORIGINAL ARTICLE – CANCER RESEARCH

Astragaloside IV inhibits progression of lung cancer by mediating immune function of Tregs and CTLs by interfering with IDO

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Abstract
Purpose Tumor cells have developed multiple mechanisms to escape immune recognition mediated by T cells. Indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme inducing immune tolerance, is involved in tumor escape from host immune systems in mice. Astragaloside IV (AS-IV), an extract from a commonly used Chinese medicinal plant *Astragalus membranaceus*, has been shown to be capable of restoring the impaired T-cell functions in cancer patients. The purpose of this study was to investigate the mechanisms underlying the anticancer properties of AS-IV.
Methods Here, we used IDO-overexpressed murine Lewis lung carcinoma cells to establish an orthotopic lung cancer model in C57BL/6 mice. Next, tumor growth was evaluated in several different treatment groups: control (saline), AS-IV, paclitaxel, and 1-methyl tryptophan (an inhibitor of IDO). We then analyzed the percentages of various immune cell subsets among the splenic lymphocytes of lung cancer mice by flow cytometry. The level of IDO was measured by real-time PCR and Western blot.
Results We showed that the growth of tumor was suppressed by AS-IV treatment in vivo. AS-IV also could down-regulate regulatory T cells (Tregs) and up-regulate cytotoxic T lymphocytes (CTLs) in vivo and in vitro. Consistent with its ability to interfere with T-cell immunity, AS-IV blocked IDO induction both in vitro and in vivo.
Conclusions The results of these studies indicate that AS-IV has in vivo anticancer activity and can enhance the immune response by inhibiting the Tregs frequency and induce the activity of CTLs, which might be related to the inhibition of IDO expression.

Keywords IDO · AS-IV · Immunotherapy · Regulatory T cells · Cytotoxic T lymphocytes

Abbreviations

IDO	Indoleamine 2,3-dioxygenase
AS-IV	Astragaloside IV
Treg	T regulatory cell
CTL	Cytotoxic T lymphocyte
3LL	Lewis lung carcinoma
1-MT	1-Methyl tryptophan

Introduction

Each tumor evolves ways to escape immune surveillance (Boon and van der Bruggen 1996), resulting in host immune system that fails to respond to tumor-associated antigens (Munn and Mellor 2007). Previous research has shown that immune escape could affect tumor dormancy versus progression, license invasion and metastasis and impact therapeutic response, which indicated that immune escape may be a central modifier of clinical outcomes (Prendergast 2008). There are various immune cell subsets, and the cell products included in the immune microenvironment, which plays a critical role in promoting and inhibiting tumor development and/or progression (Wu et al.

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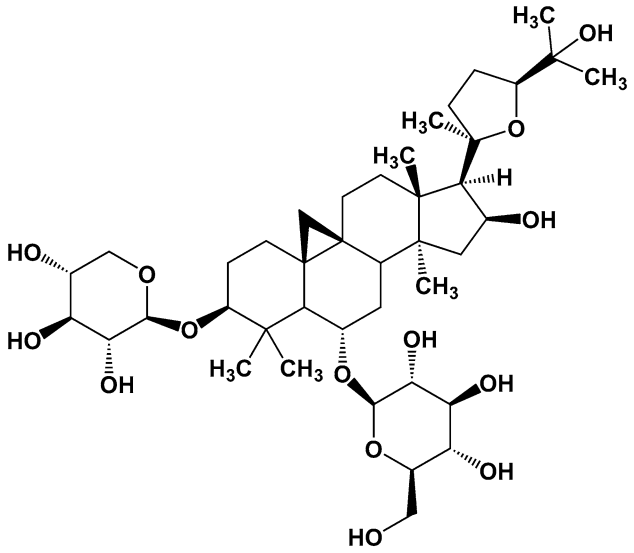

Fig. 1 Chemical structure of AS-IV. 3-0-beta-D-xylopyranosyl-6-0-beta-D-glucopyranosylcycloastragenol with molecular weight 784.98

2013). Regulatory T cells (Tregs, [$CD4^+CD25^+Foxp3^+$]) can act as a key component of acquired tolerance to tumors, so as to inhibit most types of immune responses (Chatila 2005; Sun et al. 2011). $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs) under control by Tregs and immunosuppressive cytokines, which play an important role in adaptive immunity (Chen et al. 2012), responsible for deepening the immune response (Yigit et al. 2010). CD28 is an essential co-stimulatory molecule expressed on naive T cells, the ability of $CD8^+$ T cells to protect the host declines with age, which is marked by the accumulation of dysfunctional memory cells, such as $CD28^-$ $CD8^+$ T cells (Nguyen and Weng 2010).

Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-catabolizing enzyme that induces immune tolerance in mice (Yoshida et al. 2008). In placenta, tumor-draining nodes, and primary tumors of humans, IDO expression has been observed by immunohistochemistry (Hwang et al. 2005). Enzymatically active IDO and the IDO2 proteins identified recently result in tryptophan starvation and downstream metabolite accumulation (Godin-Ethier et al. 2011). IDO could both suppress T cells directly and enhance local Treg-mediated immunosuppression, which helps create a tolerogenic milieu in the tumor and the tumor-draining lymph nodes (Boon and van der Bruggen 1996). IDO represents an ideal target for immunomodulatory drugs on account of its ability to suppress immune response (Zheng et al. 2006).

For many natural products, the mechanism of action is unknown, hampering drug development (Li et al. 2013a, b). Astragaloside IV (AS-IV) (for its structure, seen in Fig. 1), an extract from a kind of Chinese traditional herb *Astragalus membranaceus*, was proved to have strong immunoregulatory properties (Du et al. 2012). In China, AS-IV is now commonly used to treat common cold, diarrhea, fatigue, and anorexia as an immunomodulating agent in mixed herbal decoctions, and it is also prescribed to patients with cardiac diseases (Huang et al. 2012). Nevertheless, whether AS-IV possesses a potential regulatory activity in the immunosuppression ability of IDO with subsequent activation of Tregs remains unclear.

In this study, we used a mouse orthotopic lung cancer model to investigate the tumor-suppressive features of AS-IV and then analyzed the percentages of various immune cell subsets among the splenic lymphocytes of lung cancer mice treated with AS-IV, saline, or 1-methyl tryptophan (1-MT). Our further analysis showed that AS-IV reduced the proportion of Treg cells and elevated the CTL cells. We subsequently demonstrated that AS-IV could inhibit IDO expression in vitro and in vivo. Hence, we hypothesized that AS-IV mediated the relative balance of immune effector cells and suppressive immune cell populations in the tumor microenvironment by targeting IDO. The purposes of this paper are to examine the relationship between tumor-suppressive effect of AS-IV and IDO-mediated immune escape in cancer.

Materials and methods

Mice, cell lines, and plasmid

Female C57BL/6 mice (6 weeks old and weighing about 20 g) were purchased from Shanghai Laboratory Animal Resource Center and were maintained in a pathogen-free environment. Luciferase-expressing mouse Lewis lung carcinoma (3LL-luc) cell line was a gift of Longhua Hospital. The eukaryotic expression plasmid vectors pEGFP-N1 or pEGFP-N1 carrying human IDO cDNA was transfected into 3LL-luc cells by Shanghai Innovation Biotechnology Co, Ltd (named as 3LL-Luc-EGFP and 3LL-Luc-IDO, respectively). Cells were maintained in DMEM medium (Gibco Industries, Inc. Carlsbad, CA) supplemented with 10 % fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) (Invitrogen Corporation, California, USA).

Reagents

AS-IV used for this study was purchased from Pharmagenesis Inc (Beijing, China) and was dissolved in DMSO at the concentration of 100 mg/mL and then stored in −20 °C for further dilution. 1-Methyl-D-tryptophan (1-MT) was purchased from Sigma-Aldrich.

Orthotopic xenografts

For orthotopic implantation, 3LL-Luc-IDO cells (5×10^5 cells/mL) suspended in PBS containing 500 μg/mL Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) were injected into the lung of the C57BL/6 mice under anesthesia ($n = 10$ per group). Tumor progression of 3LL xenografts was detected with bioluminescent signaling during the course of study. All experiments were carried out under the approval of the Administrative Panel on Laboratory Animal Care of the Longhua Hospital.

In vivo optical imaging

Prior to in vivo imaging, the mice were anesthetized with phenobarbital sodium, and D-luciferin solution (150 mg/kg) was injected i.p. 5 min before imaging. Exposure times of bioluminescence imaging ranged from 30 s to 5 min. Fluorescence signals were simultaneously quantified using Kodak In-Vivo FX Pro on days 4, 8, and 12 after orthotopic implantation.

Splenic mononuclear cells isolate and co-culture

Spleens were teased in 3–5 mL of RPMI 1640. Cells were dissociated through a 30-μm stainless steel mesh twice and treated with erythrocytolysin. After centrifugation, the cell pellets were collected. T cells were suspended in RPMI 1640 culture medium supplemented with 10 % FCS, 100 U/mL penicillin, and 100 U/mL streptomycin. T cells and tumor cells were co-cultured in the ratio of 5:1 for 72 h.

RNA extraction and real-time RT-PCR

Total RNA was extracted from tumor issues or cells using Trizol (Invitrogen Corporation, California, USA), according to the manufacturer's instructions. Reverse transcription was performed using One Step PrimeScript® cDNA Synthesis Kit (Takara Bio Inc, Dalian, China) with an iCycler1 thermal cycler (Bio-Rad, Hercules, USA). The primer sequences were as follows: GAPDH, forward: 5′-TGTGCA GTGCCAGCCTC-3′ and reverse: 5′-CCCAATACGGCCA AATCC-3′; IDO, forward: 5′-AGATCTCGCCACCATGGC ACTCAGTAAAATATCT-3′ and reverse: 5′-GGTACC GGC CAACTCAGAAGAGCTTTC-3′.

Western blot analysis

Proteins were resolved in an SDS-PAGE gel (10 % gel) and subjected to immunoblot analysis using purified rabbit polyclonal antibody against IDO (Abgent). Cells were harvested and homogenized with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 % SDS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.02 % sodium azide, 100 g/mL PMSF, 1 g/mL aprotinin). Forty micrograms of total protein extract was separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5 % nonfat dry milk in TBS, the membrane was probed with primary monoclonal antibody specific to IDO (1:1,000; Abgent) or β-actin (1:1,000; Cell Signaling Technology) which was used as internal cellular proteins that were extracted and separated in SDS-PAGE gels. The membrane was further probed with horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG (1:2,000; Santa Cruz), and the protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Corp, Piscataway, NJ).

Statistical analysis

Each experimental value was expressed as a mean ± SD. Statistical analysis was conducted using Student's t test to evaluate the significance of differences between groups. The statistical analysis of the luciferase signal was performed using the Mann–Whitney U test to evaluate the significance of differences between groups. Kaplan–Meier method was used to generate survival curves, and the comparisons were done using log-rank tests. Statistical analysis was conducted using the GraphPad Prism5 software to evaluate the significance of difference between groups, considered as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All data points represented the mean of triplicates.

Results

AS-IV treatment suppresses tumor outgrowth

To investigate whether AS-IV could inhibit the tumor progression, we used a luciferase-expressing murine Lewis lung carcinoma (3LL-luc-IDO) cells to establish an orthotopic mouse model of lung cancer. Two initial studies demonstrated that 1-MT can limit the growth of tumors where IDO is overexpressed (Friberg et al. 2002; Uyttenhove et al. 2003), so 1-MT served as positive control in our study. AS-IV (40 mg/kg), saline (10 mL/kg), or 1-MT (100 mg/kg) was orally given daily, which lasted until mice died, and paclitaxel was i.p. given at 20 mg/mL on days 1, 2, and 3. Tumor progression was monitored by in vivo fluorescence imaging at days 4, 8, and 12 post-implantation. We found the size and fluorescence intensity of tumor in the paclitaxel, and AS-IV and 1-MT groups were smaller than the control group at 4-, 8-, and 12-d time points (*** $p < 0.001$, ** $p < 0.01$, Mann–Whitney U test) (Fig. 2a, b). Furthermore, mice treated with AS-IV showed

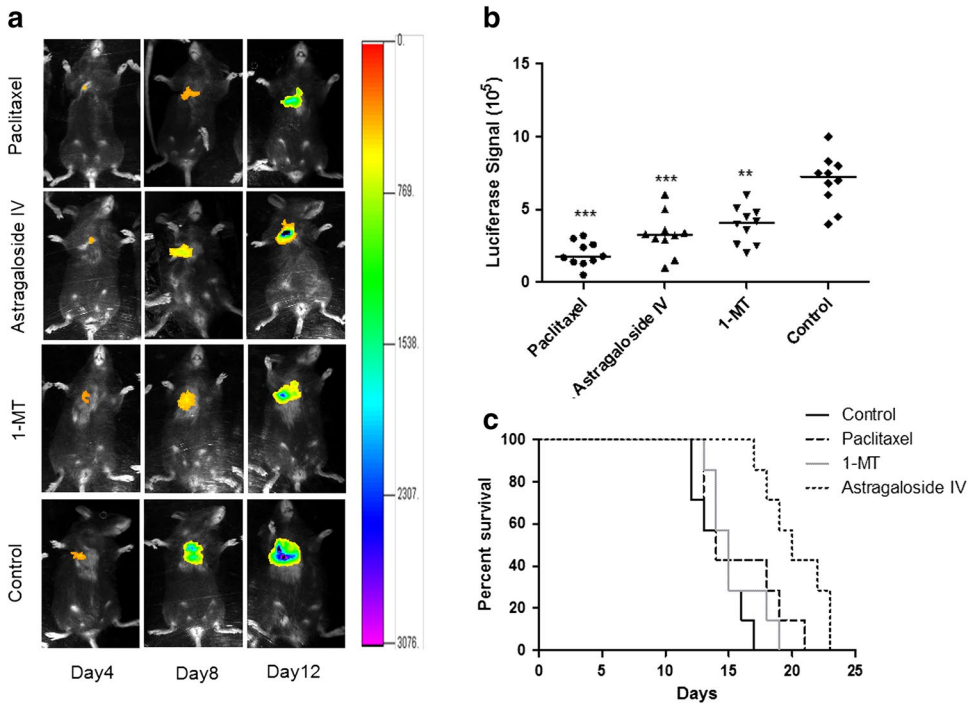

Fig. 2 Antitumor effects of AS-IV in IDO-overexpressing murine Lewis lung carcinoma orthotopic models. **a** Tumor growth in the mice was monitored by a live imaging system detecting the luciferase signal at days 4, 8, and 12. Representative images from each group are shown. **b** The intensity of luciferase signals from orthotopically xenografted tumor at day 12 was quantified by Kodak In-Vivo FX Pro. Each point in this graph represents an independent mouse. $***p < 0.001$, $**p < 0.01$, (Mann–Whitney U test), relative to control group. **c** Survival curves for 3LL-luc-IDO-xenografted C57 mice treated with saline, paclitaxel, 1-MT, or AS-IV. There was a significant difference in survival among the four groups as determined by log-rank test ($p = 0.0093$)

significantly prolonged survival ($p = 0.0093$, log-rank test) when compared to mice that were treated with saline or 1-MT (Fig. 2c). These experiments demonstrated the antitumor activity of AS-IV. However, AS-IV did not influence the cell viability of 3LL-luc-EGFP or 3LL-luc-IDO cells in MTT assays (data not shown).

Treatment with AS-IV down-regulates the percentage of Tregs and up-regulates the percentage of CTLs among the splenetic mononuclear cells in tumor-bearing mice

In the tumor cell, IDO overexpression may limit the proliferation and cytotoxic activity of cytotoxic effector T cells, providing a direct mechanism of immune escape (Prendergast 2008). To investigate the immunoregulatory activity of AS-IV in vivo, we analyzed the percentages of T-cell subsets (Tregs and CTLs) in the splenetic mononuclear cells of tumor-bearing mice treated with AS-IV, 1-MT, or saline. The establishment of lung cancer model and drug administration was described above, mice were killed at days 4, 8, and 12 post-implantation, and the splenetic mononuclear cells were isolated. In mice treated with AS-IV or 1-MT, the percentage of $CD4^+CD25^+$ Tregs was significantly decreased compared with the control group (Fig. 3a, b). Then, we investigated the expression of Foxp3 in gated $CD4^+$ T cells, the percentage of $CD4^+Foxp3^+$ T cells from spleen of mice treated with AS-IV or 1-MT in are higher than those treated with saline, and these results were consistent with the percentage of $CD4^+CD25^+$ Tregs (Fig. 3c, d). Moreover, the percentage of splenic $CD8^+CD28^+$ cells (CTLs) in mice treated with AS-IV were significantly higher than in either mice treated with 1-MT or saline (Fig. 3e, f). The data suggested that AS-IV may be able to reduce the frequency and impair the immunosuppressive function of $CD4^+CD25^+$ or $CD4^+Foxp3^+$ Tregs; meanwhile, AS-IV may enhance the immune responses via strengthening the activity of $CD8^+CD28^+$ CTLs.

Treatment with AS-IV down-regulates the percentage of Tregs and up-regulates the percentage of CTLs among the splenetic mononuclear cells co-cultured with IDO-transfected cells

To evaluate whether incubation with IDO-transfected tumor cells induced Tregs up-regulated and CTLs down-regulated, 3LL-luc, 3LL-luc-EGFP, and 3LL-luc-IDO cells were co-cultured with splenocytes from C57BL/6 mice, respectively. After cultured for 72 h, co-cultures were analyzed by flow cytometry to assess the percentages of T-cell subsets. The percentages of $CD4^+CD25^+$ T cells and $CD4^+Foxp3^+$ T cells were higher when cultured with 3LL-luc-IDO cells than cultured with 3LL-luc or 3LL-luc-EGFP cells (Fig. 4a–d). Then, we investigated the expression of $CD8^+CD28^+$ T cells, and the percentage of $CD8^+CD28^+$ cells cultured with 3LL-luc-IDO cells were higher than other two groups (Fig. 4e, f).

We next analyzed the effect of AS-IV on T-cell-mediated immunity in vitro. When 3LL-luc-IDO cells were co-cultured with splenic lymphocytes in the presence

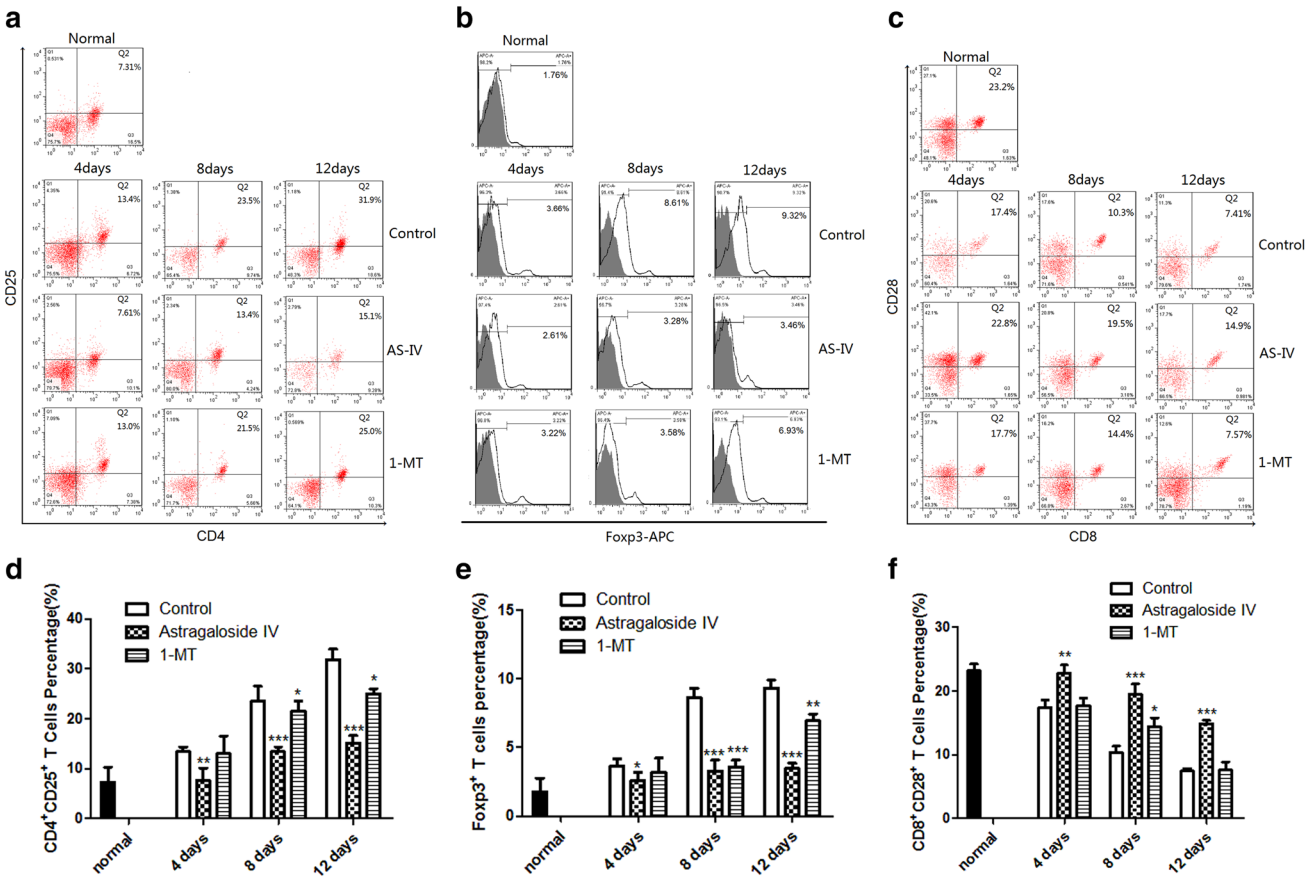

Fig. 3 Effect of AS-IV on proportion of Tregs and CTLs in splenic T cells. T cells were isolated from the spleen of C57BL/6 mice treated with saline, AS-IV, or 1-MT on days 4, 8, or 12. After staining for CD4/CD25 (**a**), CD4/Foxp3 (**c**), and CD8/CD28 (**e**) in T cells, detection was performed by FACS. The percentages of the double-positive cells of $CD4^+CD25^+$ (**b**), $CD4^+Foxp3^+$ (**d**), and $CD8^+CD28^+$ (**f**) in total T cells are shown. Statistical significance of *p* value as AS-IV or 1-MT versus control group of the same time point

of different doses of AS-IV, respectively, the expression of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ was significantly decreased compared with the same co-culture system without AS-IV (Fig. 4a–d). In contrast, the expression of $CD8^+CD28^+$ was increased after adding different doses of AS-IV (Fig. 4e, f). These regulating effects of AS-IV were more significant in 160 μg/mL AS-IV group in comparison with 80 μg/mL AS-IV group. Taken together, AS-IV appeared to be involved in modulating T-cell-mediated immunity by influencing Tregs and CTLs, which may be related to interfere with IDO.

AS-IV inhibits the expression of IDO in vitro and in vivo

Based on our observations on the antitumor functions of AS-IV in this study, intertwining with the immunoregulation effects of AS-IV on T-cell-mediated immunity, we asked whether the antitumor function of AS-IV was attributed to its suppressive effect on IDO expression. To test our hypothesis, we analyzed the expression of IDO in 3LL-luc-IDO cells with or without AS-IV added. We used Western blots to determine the effect of different doses of AS-IV on levels of IDO protein in 3LL-luc-IDO cells after incubated for 24 h. We observed that IDO protein level was decreased when 3LL-luc-IDO cells were treated with AS-IV compared with control group in a dose-dependent manner (Fig. 5a). Based on the observation that 160 μg/mL AS-IV inhibited IDO expression most efficiently, we next added this dose of AS-IV to the co-culture system of splenic T cells and 3LL-luc-IDO cells and then assessed the expression level of IDO after incubated for 24, 48, and 72 h. As shown in Fig. 5b, there is a time-dependent effect on inhibition of IDO expression by AS-IV.

To further investigate whether the inhibition of IDO by AS-IV would act the same in vivo, we implanted 3LL-luc-IDO cells orthotopically to C57BL/6 mice. Mice were treated with AS-IV, 1-MT, or saline for 12 days, and then, we examined the expression of IDO in tumor tissues. We observed that IDO mRNA and protein levels were decreased when treated with AS-IV or 1-MT compared

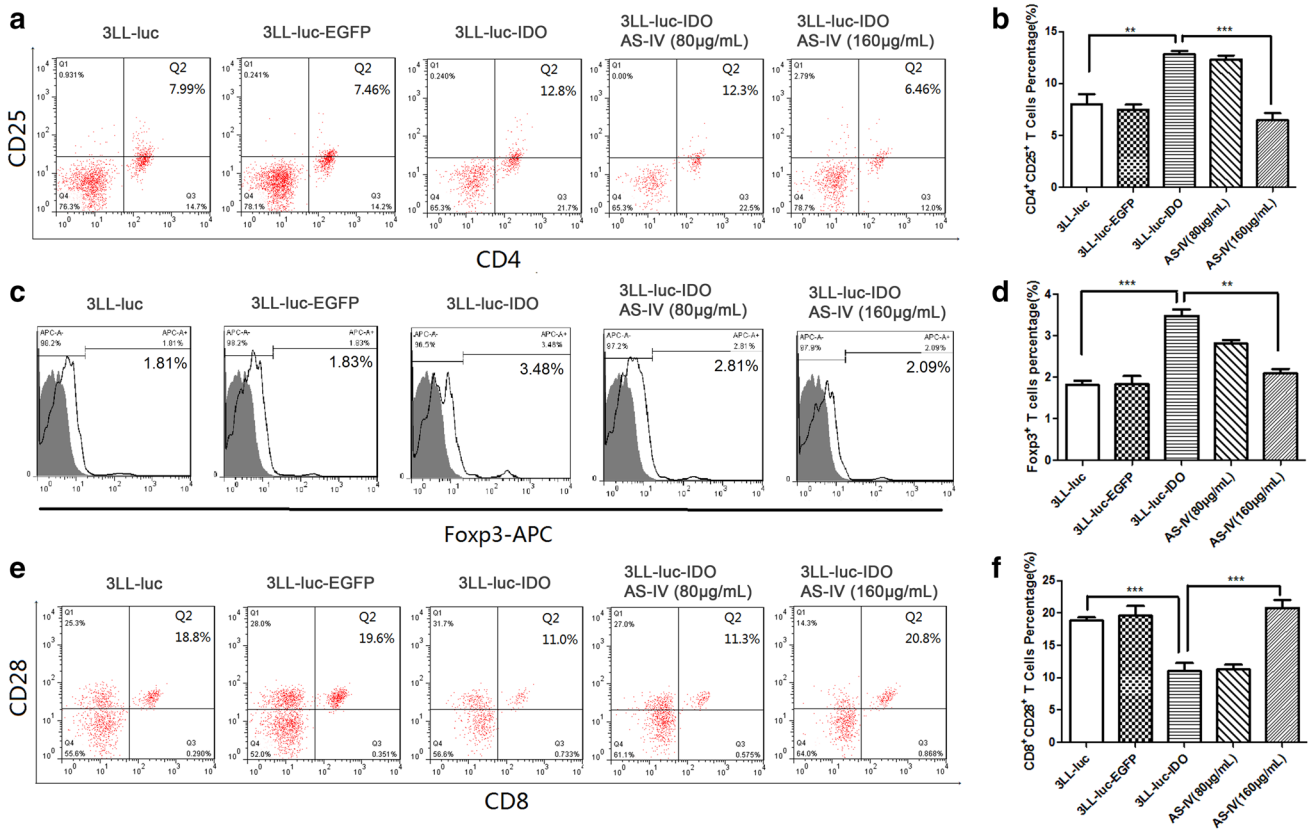

Fig. 4 The function of IDO on the proportion of Tregs and CTLs in splenic T cells co-cultured with 3LL-luc, 3LL-luc-EGFP, or 3LL-luc-IDO cells and the effect of AS-IV. After staining for CD4/CD25 (**a**), CD4/Foxp3 (**c**), and CD8/CD28 (**e**) in T cells, detection was performed by FACS. The percentages of the double-positive cells of $CD4^+CD25^+$ (**b**), $CD4^+Foxp3^+$ (**d**), and $CD8^+CD28^+$ (**f**) in total T cells are shown

with control group in mice tissues (Fig. 5c, d). Collectively, our data suggest that AS-IV suppresses IDO expression in vitro and in vivo, and it is possibly connected with the T-cell immunity and antitumor efficacy mediated by AS-IV.

Discussion

It is likely that successful immunotherapy relies on boosting immune function to overcome immune tolerance through targeting of key effectors of tumoral immune escape protecting the tumor (Muller and Scherle 2006). AS-IV is used in Chinese medicine for the enhancement of human immunity with little side effect, while the mechanisms underlying its immunoregulation properties are unclear (Li et al. 2013a, b). In this study, we have shown that, as with IDO inhibitory compounds 1-MT, AS-IV can be delivered in vivo to suppress tumor outgrowth in an orthotopic mouse model of IDO-overexpressed lung cancer. Survival following the onset of this model in the mouse can be dramatically improved by the administration of AS-IV, and it will be important to assess whether this benefit is also linked to the ability of AS-IV to inhibit IDO. However, it should be emphasized that directly extrapolating in vitro conditions and results, e.g., effective concentrations, to the in vivo system might be misleading. It is because that the in vivo system is multifactorial, AS-IV exerts its antitumor effects in an indirect manner rather than cytotoxicity.

Tumor cells transfected with IDO acquire the ability to resist immune eradication, even in preimmunized mice that had fully protective immunity (Munn 2006). Increased Treg activity facilitates tumor growth (Nishikawa et al. 2005), whereas depletion of Tregs prevents progressive growth of antigenic tumors; otherwise, the immunogenicity of tumor cells would be concealed (Ercolini et al. 2005; Sutmuller et al. 2001; Yu et al. 2005; Yang et al. 2004). IDO pathway generates tryptophan degradation as well as kynurenines and other downstream catabolites accumulation, which causes induction of Tregs and immune suppression (Fallarino et al. 2002, 2006; Munn and Mellor 2007). In the present study, we have demonstrated that AS-IV enhanced the cellular immune response in mice implanted with IDO-transfected tumor cells,

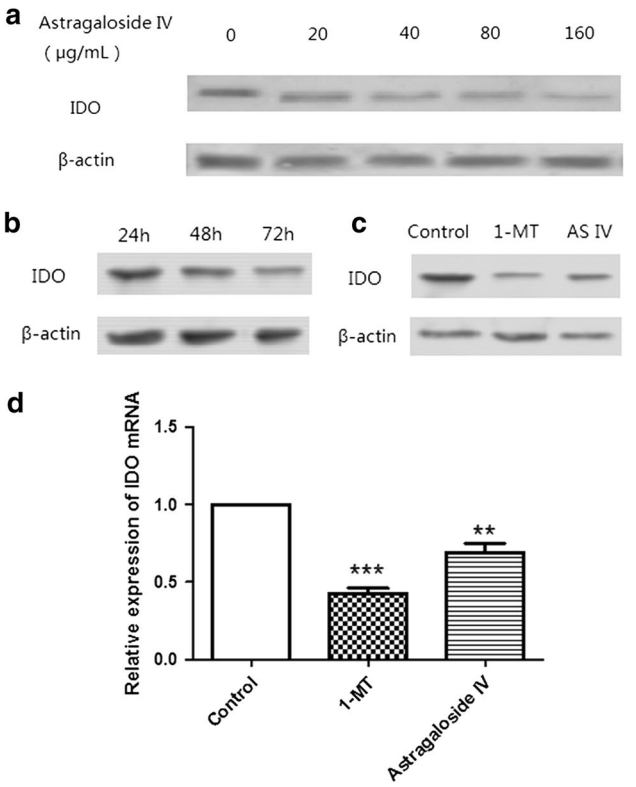

Fig. 5 AS-IV inhibits IDO expression in vitro and in vivo. **a** IDO protein expression in 3LL-luc-IDO cells co-cultured with splenic T cells under the treatment of different concentrations of AS-IV. **b** IDO protein expression in 3LL-luc-IDO cells co-cultured with splenic T cells under 160 μg/mL AS-IV treatment for different incubation times. IDO protein (**c**) and mRNA (**d**) expression in IDO-overexpressing 3LL-luc-IDO tumor tissues at day 12 post-implantation. Mice were treated with saline, 1-MT, or AS-IV. All data were presented as mean ± SD and as representative of an average of three independent experiments

which were characterized by higher expression of splenic $CD4^+CD25^+$ and $CD4^+Foxp3^+$ Tregs. Moreover, it elicited the stronger CTL activity associated with marked increase in $CD8^+CD28^+$ expression on splenocytes of mice mentioned above. To further verify whether AS-IV could mediate T-cell immunity in vitro, we isolated the single splenocyte of mice and co-cultured with tumor cells with or without IDO. As predicted, a similar result was observed that AS-IV treatment could regulate T-cell phenotypes influenced by IDO.

Based on our previous observations, it is possible that AS-IV may achieve its antitumor effect by antagonizing IDO. Herein, we showed that AS-IV could inhibit the expression of IDO in vivo and in vitro, thereby mediating activation of T lymphocyte immunity in the presence of IDO. However, further evidence was still needed to verify that IDO is the target of AS-IV. It is not clear whether AS-IV treatment could suppress tumor growth in a complete absence of IDO in the host. As we know, IDO is the rate-limiting enzyme of the tryptophan–kynurenine pathway (Stone and Darlington 2002), and the immunosuppressive effects of IDO are enhanced by both the reduction of tryptophan and the production of other metabolites involved in it (Munn and Mellor 2007). Consequently, the level of tryptophan and kynurenine is reflective of IDO enzyme activity. Although our findings revealed that AS-IV could suppress IDO expression in vivo and vitro, it will be important to further explore the change of IDO enzyme activity under the treatment of AS-IV.

Conclusions

In conclusion, we have identified that AS-IV could inhibit tumor progression and prolonged survival in a murine lung tumor model. We also demonstrated that AS-IV could efficiently increase Tregs proportion and decreased CTLs in vivo and in vitro. As part of our research on how AS-IV affects T-cell-mediated immune response and cancer progression, we demonstrated that IDO expression was downregulated by AS-IV in vivo and in vitro, it suggested that IDO might be the potential target of AS-IV, while further evidence still needed. AS-IV has superior antitumor activity relative to the IDO inhibitor 1-MT, reflect in AS-IV could prolong survival and elevate CTLs while 1-MT couldn't. AS-IV may offer an alternative, no side effect and readily accessible tool to block IDO for therapeutic purposes.

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Conflict of interest The authors declare that they have no conflict of interest.

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