

# Cucurbitacin B Controls M2 Macrophage Polarization to Suppresses Metastasis *via* Targeting JAK-2/STAT3 Signalling Pathway

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## Primary research

**Keywords:** macrophage differentiation, inflammatory, cucurbitacin B, JAK2/STAT3, metastasis

**Posted Date:** October 26th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1002730/v1>

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# Abstract

## Background

Colorectal cancer is one of the most high-risk human malignant tumors. Tumor-associated macrophages (TAMs) represent a substantial proportion of all tumor-infiltrating immune cells in the tumor microenvironment (TME), as it generally displays M1 or M2 phenotype with tumor-inhibiting or tumor-promoting activity. Cucurbitacin B has been reported to produce varieties of pharmacological effects for the treatment of cancer.

## Methods

Cell viability and proliferation were measured by CCK8 as well as colony formation assays, and cell apoptosis were analyzed by flow cytometry. The underlying mechanism was determined using western blot, microscale thermophores and immunofluorescence assays. In addition, the supernatant of cucurbitacin B-induced M2-like macrophages and colon cells were co-cultured *in vitro*, transwell and wound healing assay were employed to the related phenotypes. Two mouse model were established to investigate the anti-apoptosis and anti-metastasis of cucurbitacin B.

## Results

Herein, our study was performed to evaluate the anti-tumor and anti-metastasis effect of cucurbitacin B. It was observed that cucurbitacin B inhibited the phosphorylation of JAK2 and STAT3, and translocation from the cytosol to the nucleus. Meanwhile, we observed that cucurbitacin B bound to STAT3. Further experimentation demonstrated that cucurbitacin B reduced the polarization of M2 macrophage by down-regulating JAK2/STAT3 signaling pathway. Cucurbitacin B-induced M2-like macrophages was found to diminish the migration of CRC cells. *In vitro* study suggested that cucurbitacin inhibited the CRC cells proliferation via JAK2/STAT3 and suppressed the cell migration by suppressing M2-like macrophages polarization. Consistent with *in vitro* results, the cucurbitacin B therapy significantly inhibited tumor growth and metastasis in mice. Moreover, *in vivo* the treatment with cucurbitacin B enhanced anti-tumor immunity by regulating M2-like macrophages and promoted the expression of CD4 and CD8 in tumor microenvironment.

## Conclusion

Collectively, our results provided that cucurbitacin B might be a potential candidate agent for adjuvant therapy in the process of CRC growth and metastasis.

## Introduction

Colorectal cancer (CRC), one of the leading causes of cancer morbidity and mortality in China, with around 1.8 million newly diagnosed cases worldwide annually[1]. The high mortality rate of CRC mainly is due to metastasis in 40 percent of cases. In most cases, this is because metastatic tumor cells are resistant to therapy and evade immune responses. Accumulating evidence suggests that the alterations of immune microenvironment and epigenetic contribute to the progression and metastasis of CRC[2]. Despite significant advance in therapies for CRC, many patients show a poor response to conventional therapy, leading to subsequent recurrence and a poor prognosis, highlighting the importance in innovative therapeutic approaches.

Tumor-associated macrophages (TAMs) are a pivotal component of the tumor microenvironment, which are emerging as a factor in the development and metastasis of CRC[3, 4]. It is can divided into classically activated macrophages (M1) and alternatively activated macrophages (M2), which both have been observed in tumors[5, 6]. It is generally believed that these two types of macrophages are functionally antagonistic[7]. Alternatively activated M2 macrophages promote the occurrence and development of tumors by involving in moderating inflammatory responses, promoting angiogenesis and contributing to tissue remodeling[8]. In respond to T helper type 2(Th2) cytokines such IL-4 and IL-13 results in alternative activation(M2), characterized by increase of mannose receptor (CD206), increased arginase-1 (Arg-1), found in inflammatory zone (Fizz-1), chitinase-like 3(Ym-1), Chemokine (CCL2)[9]. More importantly, increasing the number of M2 macrophages is a vital marker of CRC and is directly associated with poor clinical prognosis[10, 11]. Thus, it is paramount to extensively and intensively understand the relationship between macrophage infiltration and colorectal patients' tumor progression, for the development of effective therapeutic strategies.

JAK/STAT pathway serves as multiple regulatory roles to modulating immune function, cell growth, differentiation and death[12–14]. Structure activation of JAKs contributes to phosphorylation of the signal transducer and activator of transcription (STAT) family[15, 16]. STAT3 signaling is closely correlated with in building a protumor genic inflammatory microenvironment[17]. STAT3 activation in macrophages has been shown to enhance proliferation and survival of macrophage, and potentiate pro-tumor functions such as immune tolerance and extracellular matrix remodeling inhibition of colon cancer cells[18]. Therefore, STAT3 inhibition is considered to be effective for patients with advanced malignant tumors.

Cucurbitacin B is a main active monomer of *Cucurbitaceae*, and has been widely used in the treatment of malignant tumors[19–21]. *In vivo* and *in vitro* experiments have shown that cucurbitacin B inhibited the proliferation of multiple cancers via NF- $\kappa$ B, JAK/STAT, PI3K/AKT, and MAPK/ERK signaling pathways[22, 23]. To date, cucurbitacin B is a highly promising anti-tumor drug. However, the specific molecular mechanism under of cucurbitacin B in CRC remains unclear.

Traditional herb medicines serve as growing vital roles in inhabitation and treatment of multiple cancers[24]. Here, we researched the anti-tumor and anti-metastasis effects of cucurbitacin B on the CRC, and we revealed the potential molecular mechanisms. *In vitro* assays results suggested that cucurbitacin

B might inhibit the proliferation of CRC by JAK/STAT3 signaling pathway. According to the gap, herein, IL-4/13 or LPS was used to stimulate macrophages polarizing to M2 or M1 phenotype. The M2 polarized macrophages were co-cultured with CT-26 to develop a M2-like TAMs macroenvironment. The effects of cucurbitacin B on the metastatic behavior of CT-26 and HCT 116 in the co-culture system were investigated. In addition, the *in vivo* anti-cancer effects of cucurbitacin B were investigated in a subcutaneous xenograft tumor mice model. This study provided a novel mechanism of disrupting the M2 polarization and promoted the M1 polarization-induced signaling to inhibit the tumor growth, which can be utilized to develop therapeutic applications of cucurbitacin B as an anti-metastatic therapy for colon cancer.

## Results

### Cucurbitacin B suppressed the proliferation of colorectal cancer cell

We performed a CCK8 assay to investigate the ability of cucurbitacin B in inhibiting tumor proliferation. Cucurbitacin B effectively inhibited the viability of HCT116 and CT26 cells in dose-dependent manner, with IC<sub>50</sub> values of 435.70 nM and 364.20 nM respectively. To further investigate whether cucurbitacin B induced apoptosis, the percentage of Annexin V apoptosis cell was analyzed by flow cytometry with Annexin V/PI staining. HCT116 and CT26 cells were treated with cucurbitacin B at different concentrations (400 nM and 800 nM) for 48 h. The apoptosis of HCT116 and CT26 cells was dramatically increased after treatment with cucurbitacin B. Next, colony formation assay revealed that cucurbitacin B prohibited colonies in concentration-dependent manner, the significant different between control and cucurbitacin B group. Here, we also found that increase in apoptosis concomitant with the changes in expressions level of cleaved caspase-3, Bax and Bcl-2.

As a result, the above data demonstrated that cucurbitacin B exerted anti-tumor effects on CRC.

### Cucurbitacin B inhibited JAK2/STAT3 signaling

We next sought to determine conducted the expression of JAK2 and STAT3 in CRC cells treated with cucurbitacin B. Figure 2a shows significantly decrease on phosphorylated JAK2 and STAT3 in CRC cells treated with cucurbitacin B.

We next determined to investigate whether IL-6-triggered STAT3 activation differed between control and cucurbitacin B treatment. We therefore examined the expression level of total and phosphorylated STAT3 in CRC cells treated with cucurbitacin B. As anticipated, exposure of CT-26 and HCT116 cells to 25 ng/mL lead to increased nuclear level of phosphorylated STAT3 compared with unstimulated CT-26 and HCT116, whilst cucurbitacin B can suppressed this translocation. Mechanistically, to further validate the interaction between cucurbitacin B and STAT3, fluorescence measurement and microscale thermophoresis (MST) were performed. We found cucurbitacin B bound to stat3 ( $K_d = 39.75 \mu\text{mol/L}$ ) (Figure 2b). Moreover, the double IF staining of p-STAT3 was conducted to further confirm the importance

of JAK-2/STAT3 signaling pathway. According to Figure 2c, that the nuclear p-STAT3 level was increased treated with IL-6 and decreased when cells were treated with both IL-6 and cucurbitacin. Collectively, these results supported that the inhibitory effect of cucurbitacin B on the normal or IL-6-triggered p-STAT3 in CRC cells.

## **Cucurbitacin B inhibited Macrophages switching to M2 phenotype**

Multiple mechanisms of immune suppression can arise in tumor to facilitate the metastasis of cancer[25]. It is generally accepted that M2 phenotype is crucial in growth of tumor. To future order to investigated whether cucurbitacin B is involved in macrophage polarization,we performed flow cytometry analysis and RT-PCR assays. First of all, RAW264.7 and THP-1-derived macrophage cells were exposed with or without cucurbitacin B investigate how cucurbitacin B affected the distribution of M2 polarization. It is well known, CD206 is a marker for activated M2 macrophage[26]. It is well known that M2 polarization of macrophages are activated by cytokines such as IL-4 and IL-13. As shown in Figure 3a and 3b, on the one hand, stimulation of IL-4/IL-13 (without cucurbitacin B) significantly increased the expression of CD206 marker ( $p < 0.05$ ). Afterwards, cucurbitacin B has shown to be able to suppress the IL-4/IL-13-induced expression of chitinase 3-like 3 (Ym-1), inflammatory zone (Fizz-1), arginase 1 (Arg-1), and CCR-2 (Figure 3c and 3d) ( $p < 0.05$ ).

Collectively, these observations suggest that cucurbitacin B inhibited polarization of TAMs to M2 phenotype in RAW264.7 macrophages.

## **The JAK-2/STAT3 signaling pathway involved in cucurbitacin B-mediated inhibition of M2 polarization**

Previous studies indicated the JAK2/STAT3 pathway plays a vital and inflammatory mediator in macrophage polarization. The JAK-2/STAT3 signaling pathway has shown to participate in IL-4/IL-13-induced TAMs polarization. Herein, the phosphorylation status of JAK-2/STAT3 in IL-4/IL-13-induced RAW264.7 and THP-1-derived macrophages with or without cucurbitacin B was tested using western blotting assay. As described in Figure 4a, in RAW264.7 macrophages, the phosphorylation status of JAK-2/STAT3 was enhanced after treating cells with IL-4/IL-13. This increased phosphorylated level of JAK-2 and STAT3 was significantly inhibited by co-treatment with cucurbitacin B, suggesting that JAK-2/STAT3 signaling pathway might participate in cucurbitacin B-mediated inhibition of M2-like TAMs polarization. Therefore, collectively, these findings support the hypothesis that JAK-2/STAT3 signaling pathway participated in cucurbitacin B-mediated inhibition of M2-like TAMs polarization.

## **Cucurbitacin B inhibited the migration induced by inhibiting M2 polarization of TAMs**

It is well known that M2-like macrophage polarization is a major obstacle to effective anti-metastasis immune response. According to our above study, cucurbitacin B can suppressed M2-like macrophage

polarization. Therefore, collecting condition medium from cucurbitacin B-stimulated M2 macrophages to investigate the effect on CT-26 cells' migration and invasion. Herein, CT-26 and HCT116 were co-cultured with IL-4/IL-13-stimulated RAW264.7 and PMA induced THP-1 cells, and then the capacity of cell migration and invasion to explore the effects of M2-polarized TAMs on capacity of migration and invasion of CT-26 and HCT116. The capacity of CT-26 and HCT116 migration and invasion was detected by transwell assay and wounding heal assay. The results suggest that M2 polarized TAMs promoted the cell invasion abilities (Figure 5a) of CT-26 and HCT116. The 24 h wounding healing assay was also performed to further confirm the migration ability of colon cancer cells. Cucurbitacin B gradually decreased the healing rate of CT-26 infiltrated with M2 polarized TAMs ( $p < 0.05$ ) (Figure 5b). These findings together revealed that cucurbitacin B could inhibit the migration and invasion capacity of colon cancer cells induced by M2-like TAMs polarization.

## **Cucurbitacin B inhibited tumor growth and metastasis *in vivo***

To determine the effects of cucurbitacin B on CRC growth and metastasis *in vivo*, we injected luciferin labeled CT-26 cell in C57BL/6 and BALB/c mice receptively. Figure 6a and 6b showed that treatment of cucurbitacin B at dose of 0.5 and 1 mg/kg significantly inhibited tumor growth, which was reflected by the decrease of the volume and the weight of tumor, and the final volume and the weight of tumor from mice administrated with cucurbitacin B were significantly lower than those from control mice. In conjunction, no significant difference in body weight was observed between control and the cucurbitacin B-treated groups (Figure.6c) ( $p > 0.05$ ). The morphological structure of tumors was observed by HE staining. This is in accordance with the results in Figure 6d, 0.5 and 1 mg/kg cucurbitacin B increased number of necrotic cells in the tissues, and the infiltration of large number of inflammatory cells into tumor tissues.

Ki67 and caspase-3 are major proteins taking part in the regulation of tumor growth. As expected, the level of proliferation and apoptosis measured by Ki67 and caspase-3 staining (Figure 6d) were increased in administration of cucurbitacin B compared to control ( $p < 0.05$ ), indicating that administration of cucurbitacin B at the dose of 0.5 and 1 mg/kg significantly inhibited the expression of Ki67 and increased caspase-3 in tumor tissues. These pathological analyses indicated that the administration of cucurbitacin B effectively induced the cell apoptosis, and thus prevented the tumor growth.

To verify the applicability of the cucurbitacin B therapy to CRC, the effects upon colon cancer metastasis were appraised using a colon cancer lung metastasis model. As results, the number of pulmonary tumor nodules dramatically upregulated after intravenous injection of CT-26 cells. However, cucurbitacin B at dose of 0.5 and 1 mg/kg for 2 weeks prominently decreased the bioluminescence accumulation (Figure 6f). This data supported the notion cucurbitacin B treatment inhibited CT-26 cell metastasis in BALB/c mice.

# Cucurbitacin B regulated M2-like polarization and promoted M1-like polarization in C57BL/6 murine colon cancer model

We further verify whether cucurbitacin B suppressed the progression of colon cancer by regulating macrophages. The colon sections from different groups were stained with the M2-macrophage marker CD206 by IHC staining. The results are shown in Figure 6a, administration of cucurbitacin B significantly decreased the expression of CD206 in a dose-dependent manner when compared to control groups ( $p < 0.05$ ). In agreement with this, Figure 6b indicated that the expression of CD206/CD11c were remarkably suppressed after cucurbitacin B treatment in mouse spleen.

It is reported that macrophage can produce cytokines that participated in CD4+T and CD8+T cell activation. To further investigate the effect on macrophage by cucurbitacin B, we examined the infiltration of the T cells population in the CT-26 allograft after cucurbitacin B treatment by flow cytometry. As shown in Figure 6c, significant increase in the population of CD4+T and CD8+T cells were observed after cucurbitacin B treatment. ( $p < 0.05$ .) These findings suggested that cucurbitacin B could relief TAM-mediated immunosuppression.

## Discussion

As reported, cucurbitacin B exhibited anti-tumor and anti-metastasis effect in diverse cancer such as lung cancer, pancreatic cancer, neuroblastoma cancer and colon cancer *in vitro* and *in vivo* [27–29]. In continuation to characterize that cucurbitacin B exert anti-tumor effect via various pathway, this study aimed to explore the effect of cucurbitacin B on tumor growth and metastasis *in vitro* and *vivo*. Consistent with the previous findings, we observed the anti-tumor effects of cucurbitacin B on CRC cells CT-26 and HCT116 cells *in vitro*. In our study, we validated that cucurbitacin B markedly inhibited cell viability in CRC cells. At the same time, our data also showed that cucurbitacin B had a similar impact on apoptosis and colony formation of CT26 and HCT116 cells. In addition, these results demonstrated cucurbitacin B suppressed the growth of CRC cells by apoptosis. We then find the cucurbitacin B decreased the expression levels of phosphorylated STAT3 in a concentration-dependent manner. Meanwhile, our data also showed that cucurbitacin B could inhibit the IL-6-induced phosphorylation of STAT3. Particularly, a strong binding affinity was observed between cucurbitacin B and STAT3 by microscale thermophoresis analysis. Moreover, cucurbitacin B diminish nuclear accumulation of p-STAT3 by immunofluorescence analysis. Altogether, these results demonstrated suggested that cucurbitacin B could target STAT3 in CRCs.

Macrophages, one of the main immune cells in TMA, have been validated to correlated with the growth and metastasis of various type of tumor[30]. Within the progression of tumor, M1 activation and M2 activation in tumor elicit anti-tumor and pro-tumor functions respectively. Clinically, M2-like TAM are one of the amplest cell types in diverse solid tumor and an important role to play in modulating tumor progression and metastasis[31]. It has been proved that large numbers of macrophages infiltrating into colon adenocarcinoma tissues were related to the poor patient prognosis[32]. Therefore, reprogramming

of M2-like macrophage toward the antitumoral might be potential new therapeutic targets. More and more studies have indicated the mechanism of monomeric components of traditional Chinese medicine exert anti-tumor effect by modulating M1 or M2 macrophage polarization[33, 34].

Despite it has been reported that cucurbitacin B gains great attention as a natural product which exert anti-tumor immunity, the underlying mechanism of its macrophage regulation is still poorly unexplored[35]. The remarkable inhibition of M2-like RAW264.7 and THP-1-derived macrophages polarization by the administration of cucurbitacin B was observed in this study.

The M1 and M2 phenotypes were aim to define macrophages, however the specific markers were ultimately expression on monocytes. Our data showed lower expression of CD206 (M2) and the M2 markers (Ym-1, Fizz-1, Arg-1, IL-10) extremely diminish after cucurbitacin B treatment. To date, it has been proved that CD206<sup>+</sup>M2 TAMs increase cancer progression by STAT3 activation, inducing and maintaining a microenvironment[36]. Therefore, JAK2/STAT3 signaling in TAM is a crucial component of response to M2-like TAMs macrophages[37]. Herein, the role of JAK2/STAT3 signaling pathway in inhibiting M2-like TAMs macrophages induced by cucurbitacin B was investigated. The findings in this study show that cucurbitacin B-induced inhibition of M2 decreased the phosphorylation status of JAK2 and STAT3. We therefore conclude that JAK2 /STAT3 participate in the progression of cucurbitacin B-induced inhibition of M2-like macrophages.

CRC is considered as an inflammation-related cancer, likewise one of the most common reasons of cancer-associated deaths worldwide[38, 39]. Therefore, we inferred that cucurbitacin B was more likely to alter the tumor microenvironment, inhibiting M2 polarization, and thus inhibiting tumor metastasis. A co-culture system was performed to explore the effect of cucurbitacin B-stimulated M2 phenotype macrophages on migration *in vitro*. Herein,we were surprised to find that cucurbitacin B suppress M2 macrophage polarization, which result in inhibiting migration and invasion of colon cancer cell in our established *in vitro* colon TAM model. The findings in this study revealed that cucurbitacin B suppressed M2-like macrophage and to further enhance the anti-metastasis *in vivo*.

*In vivo* study, cucurbitacin B has shown to inhibit the tumor growth and metastasis in C57BL/6 and BALB/c murine model respectively, and be functioned as an apoptosis inducer, as the evidence of increased Ki67 and caspase-3 expression levels in cucurbitacin B-administrated murine groups. However, the potential mechanisms of inducing the cell apoptosis and inhibiting the cell proliferation still need to be further investigated via *in vivo* study. C57BL/6 murine bowel cancer model was employed in this study. Interestingly, macrophages were identified in TME, including M2 macrophages aimed at driving tumor progression. Due to the effect of cucurbitacin B on macrophage polarization, the effects of cucurbitacin B on altered TAMs polarization in tumor tissues *in vivo* were investigated. We found that under treatment of cucurbitacin B, the expression of M2 marker (CD206) diminish in TAM. TMA can modulate the killing effect of T cells on tumor cells. Increasing studies show that the inhibition of M2 polarization will ultimately induce the activation of CD4<sup>+</sup> and CD8<sup>+</sup>T cell and its immune response[40, 41]. In this study, we demonstrated that cucurbitacin B induced more T cell infiltration into the tumors.



Cumulatively, these results suggest TAMs emphasized the anti-tumor effect of cucurbitacin B, especially via the JAK2/STAT3 signaling pathway, provided a potent strategy to regulate colon cancer cells growth and metastasis, and gave a more rational and long-term support for the clinical application of cucurbitacin B.

## Conclusion

Taken together, our results revealed that cucurbitacin B suppressed CRC cell growth respectively by inhibiting JAK2/STAT3 signaling. Furthermore, this study presented an important role of the positive feedback loop between CRC cells and TAMs in tumor associated environment. The anti-metastasis effect of cucurbitacin B was targeting inhibiting M2-like TAMs polarization via JAK2/STAT3 signaling pathway. This study provided a novel insight regarding the potential therapeutic application of cucurbitacin B as an anti-metastatic therapy for colon cancer, and possibly for other cancers as well.

## Materials And Methods

**Cell lines and cell culture** Mouse macrophage cell line RAW 264.7, the human monocyte cell line THP-1, human colon cell line HCT116 and mouse colon cell line CT26 were kindly provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). RAW264.7 Cell were maintained in DMEM, while THP-1, HCT116 and CT26 were maintained in RPMI-1640. All cultures were supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. The macrophages were polarized into M2 by 20 ng /mL IL-4/IL-13 for 24 h. Phorbol-12-Myristate-13-acetate (PMA) was taken to utilized to differentiate THP-1 cells.

**CCK-8 assays** CT-26 and HCT116 were seed in a 96-well plate with a density of  $5 \times 10^3$ . Cell Viability Assay was determined using CCK8 in accordance with the manufacture' s instruction. The absorbance was measured at 450 nm with a microplate reader.

**Flow cytometry** RAW264.7 and THP-1 cells were stained with the following fluorochrome-conjugated antibodies: anti-CD206-PE (Biolegend, USA), anti-CD86-FITC (Biolegend, USA). Single-cell suspension from tumors were performed using mouse tumor dissociation kit (Miltenyi Biotec, Germany). Stained cells were analyzed by FACS. Data analysis was performed using FlowJo software.

**Colony formation** CT-26 and HCT116 were seed in a 12-well plate with a density of  $1 \times 10^3$ . The fresh medium was changed every day to keep the cell growing. After 10 days, the colonies were fixed with 4% paraformaldehyde and dyed with crystal violet (Beyotime, China).

**RT-PCR analysis** RNA was firstly isolated from cell using MolPure<sup>®</sup> cell RNA Kit (Yesen, China). mRNA expression was performed using SYBR Green PC Master Mix (Yesen, China). Reaction was carried out using RT-PCR kits (Applied Biosystems, Canada). Expression of genes were analyzed as  $RQ = 2^{-\Delta\Delta Ct}$ . Primer sequences used for RT-PCR are listed in Table 1.

**Microscale Thermophoresis** The interaction between Cucurbitacin B and STAT3 was detected by Microscale Thermophoresis using the Nano Temper Monolith NT.115 instrument. Each measurement consists of 16 traction mixtures where fluorescent-labeled stat3, and two-fold diluted cucurbitacin B ranging from 100 $\mu$ M to 3.1nM was used. The MO. affinity Analysis v2.3 software was used to measure the  $K_d$ .

**Wound healing assay** CT-26 and HCT116 cells were planted in a 12-well plate ( $5 \times 10^6$ ) and grown until 80% confluent, and a wound was made by dragging a plastic pipette tip across the cell surface. Then wound healing image was photographed again the next day using microscope. The area of the wound was measured with image J software.

**Migration assay** The migration assay was conducted in a 24-well cell culture chamber employing inserts with 8- $\mu$ m pores (Corning, NY, USA). Inserts that contain  $2 \times 10^5$  CT-26 and HCT116 cells were transferred to wells containing  $5 \times 10^5$  M0 macrophages, M2 macrophages cultured with or without cucurbitacin B for 24 h. A cotton swab cell was removed on the top well. After fixation in 4% paraformaldehyde, the filters were stained with crystal violet for 15 min.

**Western blot analysis** CT-26, HCT116, RAW264.7 and THP-1 cells were lysed in RIPA buffer (Thermo, MA, USA). The cell lysates were separated on 10% or 12.5% SDS-PAGE gels. The antibodies used were against JAK2, p-JAK2, STAT3, p-STAT3. (Cell Signaling Technology, USA).

**Immunofluorescence staining** Cells that treat with above way were fixed with 4% paraformaldehyde (Beyotime, China) for 30 min and were incubated with STAT3 antibody. The image was pictured under the confocal microscope.

**Cell apoptosis assay** Cell apoptosis was evaluated using an Annexin V- FITC Kit (Biolegend, USA). Cells were analyzed using a CytExpert flow cytometer (Beckman Coulter, USA).

**C57BL/6 and BALB/c murine colon cancer model** *In vivo* experiment was conducted in compliance with the relevant laws and institutional guidelines. In C57BL/6 model: CT-26 ( $8 \times 10^5$ ) in 0.2 mL were injected subcutaneously into the flank of each mouse. After 1 d, mice were intraperitoneally injected administrated with 0.2 mL of cucurbitacin B (0.5 mg/kg) and 0.2 mL of Cucurbitacin B 1 mg/kg) for 25 d according to previous study[20]. Control group received equal volumes of normal saline (NS). On day 14 the animals were euthanized.

Table 1

Mouse Ym-1	Forward	5'-AGAAGGGAGTTTCAAACCTGGT-3'
	Reverse	5'-CTCTTGCTGATGTGTGTAAGTGA-3'
Mouse Fizz1	Forward	5'-AGTCCCTGCCCTTTGTACACA-3'
	Reverse	5'-CGATCCGAGGGCCTCACTA-3'
Mouse Arg-1	Forward	5'-CTCCAAGCCAAAGTCCTTAGAG-3'
	Reverse	5'-GGAGCTGTCATTAGGGACATCA-3'
Mouse IL-10	Forward	5'-CTTACTGACTGGCATGAGGATCA-3'
	Reverse	5'-GCAGCTCTAGGAGCATGTGG-3'
Human Ym-1	Forward	5'-TGCCACCTCCAGTCCAGTGA-3'
	Reverse	5'-ATGCCGTAGAGCGTCACATC-3'
Human Fizz1	Forward	5'-CGTCCTCTTGCCTCCTTCTC-3'
	Reverse	5'-ACAAGCACAGCCAGTGACAG-3'
Human Arg-1	Forward	5'-TGCCACCTCCAGTCCAGTGA-3'
	Reverse	5'-GCATCCAGCTTGACCAGAGA-3'
Human IL-10	Forward	5'-GATCTCCGAGATGCCTTCAG-3'
	Reverse	5'-ATCGATGACAGCGCCGTAGC-3'

CT-26 cells/effluc cells ( $1 \times 10^6$ ) intravenously were injected into BALB/c the for a tumor progression study. Luminescent tumor images were monitored after in the intraperitoneal injection of Luciferin for 10min and detected by a Xenogeny IVIS Lumina II imaging system.

**Immunofluorescent staining** Tumor tissues were made into slices. Then, all tumor slices were incubated with anti-Ki67 antibody, anti-Caspase-3 antibody and anti-CD206 antibody (1:200) (Cell Signaling Technology, USA), followed by incubation with secondary antibodies. Images were taken through a light microscope.

## Statistical analysis

Values are expressed as the mean  $\pm$ SD. Data were analyzed with GraphPad Prism software 8.0. Statistical analysis was performed by Unpaired Student's t-test (two-tailed) and one-way ANOVA test.  $P < 0.05$ (\*) was considered statistically significant.

## Abbreviations

Colorectal cancer CRC, Tumor-associated macrophages TAMs, classically activated macrophages M1, alternatively, activated macrophages M2, chitinase-like 3 Ym-1, Found in inflammatory zone Fizz-1, increased arginase-1 Arg-1, the signal transducer and activator of transcription STAT.

## Declarations

### Author contributions

Each author is expected to have made substantial contributions to the conception Z.Y, Z.H.Y, Z.B design of the work, Y.Y the acquisition, analysis, Z.H.R, Z.D.Y interpretation of data, Z. Y have drafted the work or substantively revised it.

### Funding

This work was supported by the National Natural Science Foundation of China No. 81803926

### Availability of data and materials

All other data are included within the Article or Supplementary Information or available from the authors on request.

### Ethics approval and consent to participate

The animal studies were approved by Shanghai University of Traditional Chinese Medicine (Approval No. P2SHUTCM201120008)

### Consent for publication

We would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

### Competing interests

The authors declare no competing interests.

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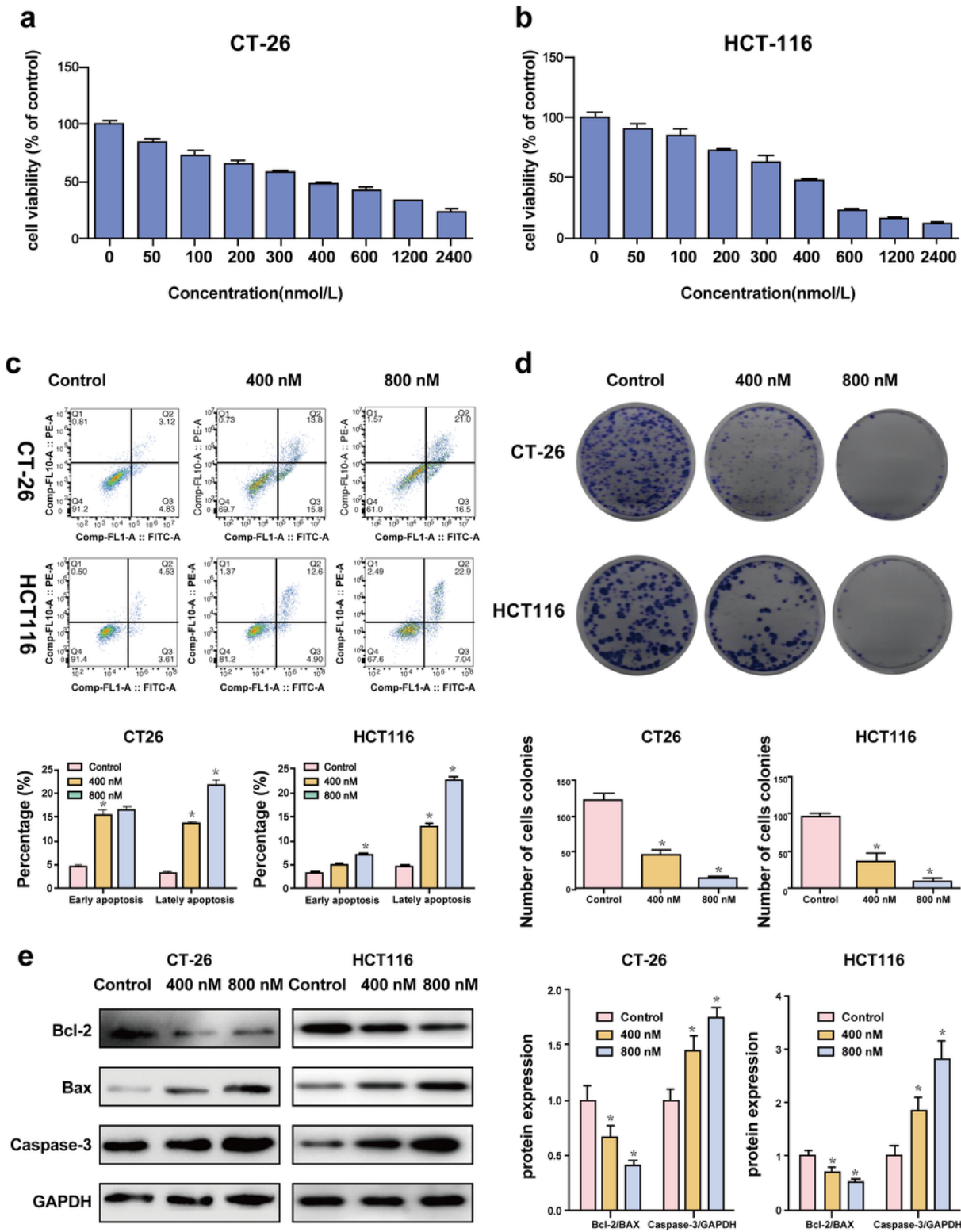
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## Figures

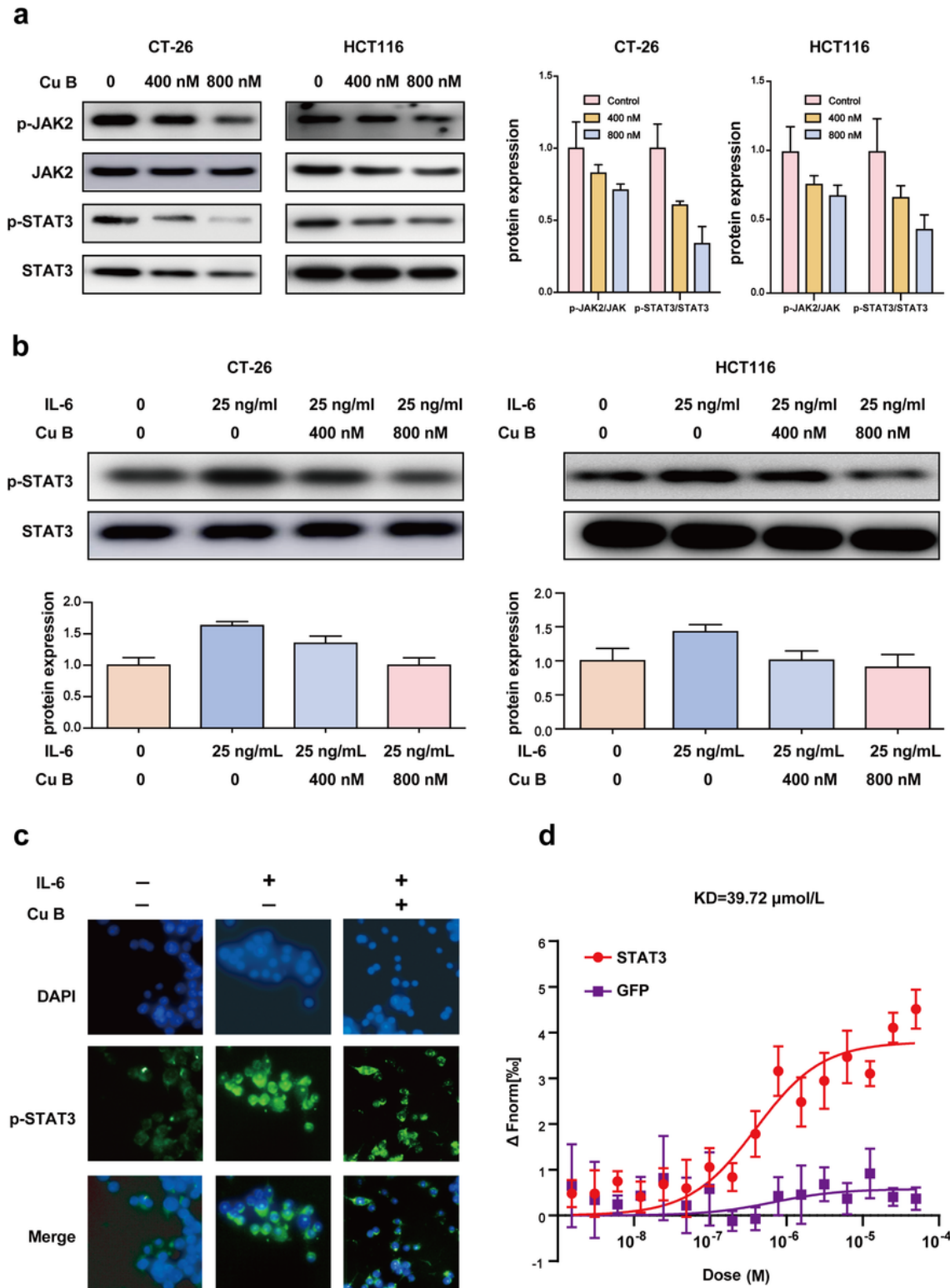


**Figure 1**

The anti-tumor effect of cucurbitacin B in CRC cells (a) Cell were incubated with increasing concentration of cucurbitacin B for 48 h and detected using CCK8 (b) CT26 and HCT116 cells were treated with 50 nM and 100 nM of cucurbitacin B for 24 h, stained with Annexin V and PI, and analyzed by flow cytometry. (c) The cloning formation of CT-26 and HCT116 cells treated with different concentration of cucurbitacin B for 10 day. (d) Protein were prepared from cells treated with or without 50 nM or 100 nM cucurbitacin B



for 48 h, and then subjected to Western blotting using antibodies as indicated. (n=4, \*p<0.05 vs control group,)



**Figure 2**

Cucurbitacin B downregulates JAK2/STAT3 signaling pathway. (a) The expression of p-JAK2, JAK, p-STAT3 and STAT3 in CT-26 and HCT116 cells was analyzed by western blot after treatment by cucurbitacin B. (b) CT-26 and HCT116 cells were treated with cucurbitacin B for 48 h. For stimulation,

cells were exposed to IL-6 (25 ng/mL) for 30 min at the end of incubation period, and cell Lysates were subjected to immunoblotting to detect the distribution of p-JAK2, JAK, p-STAT3 and STAT3. (c) Treated cells were stained with p-STAT3 and Hoechst. One representative image of three experiments is shown. (b) Microscale thermophoresis (MST) were performed to validate the interaction between CCB and STAT3. (n=4, \*p<0.05 vs control group, #p<0.05 vs IL-6-induce CRC cells group)

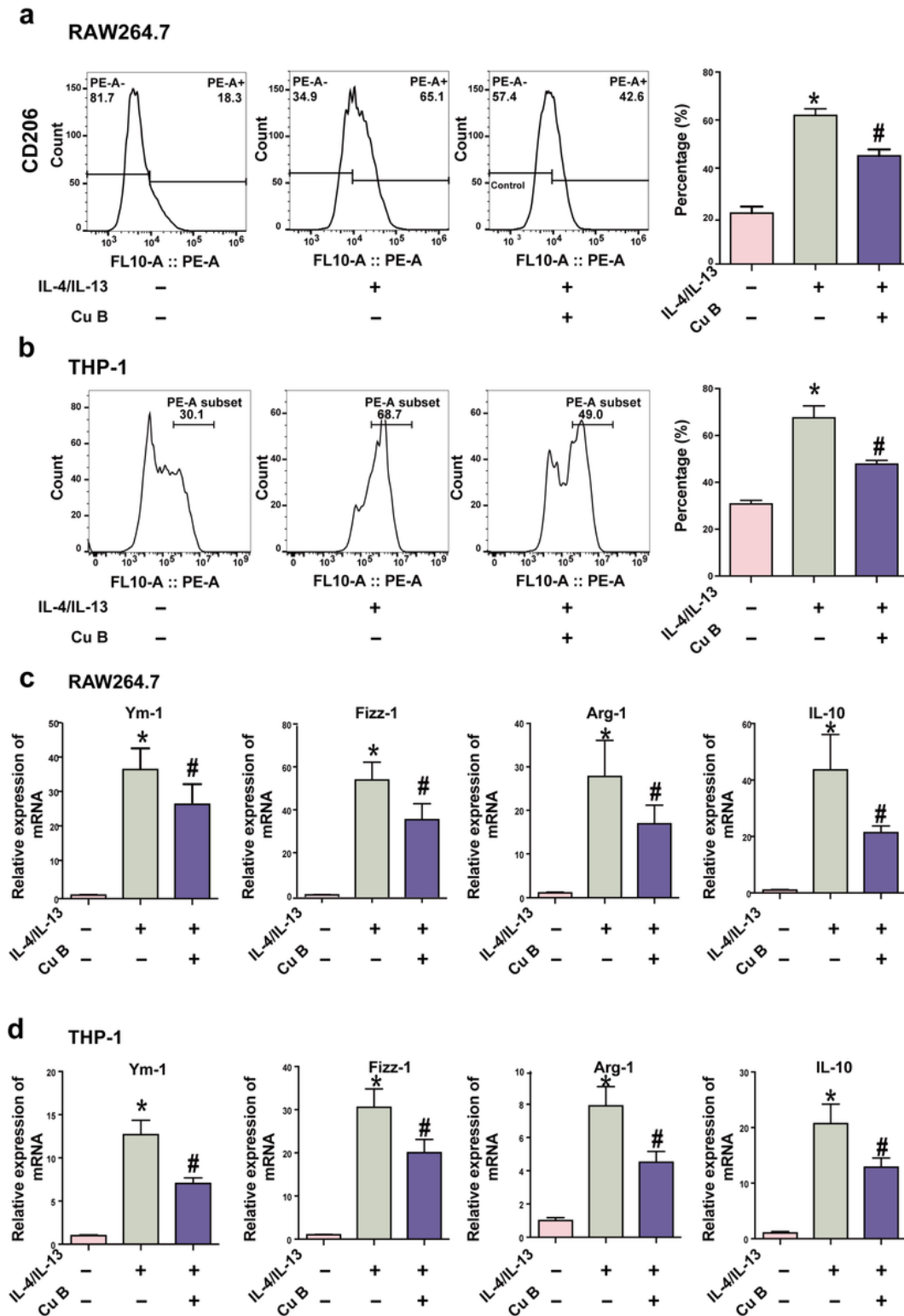
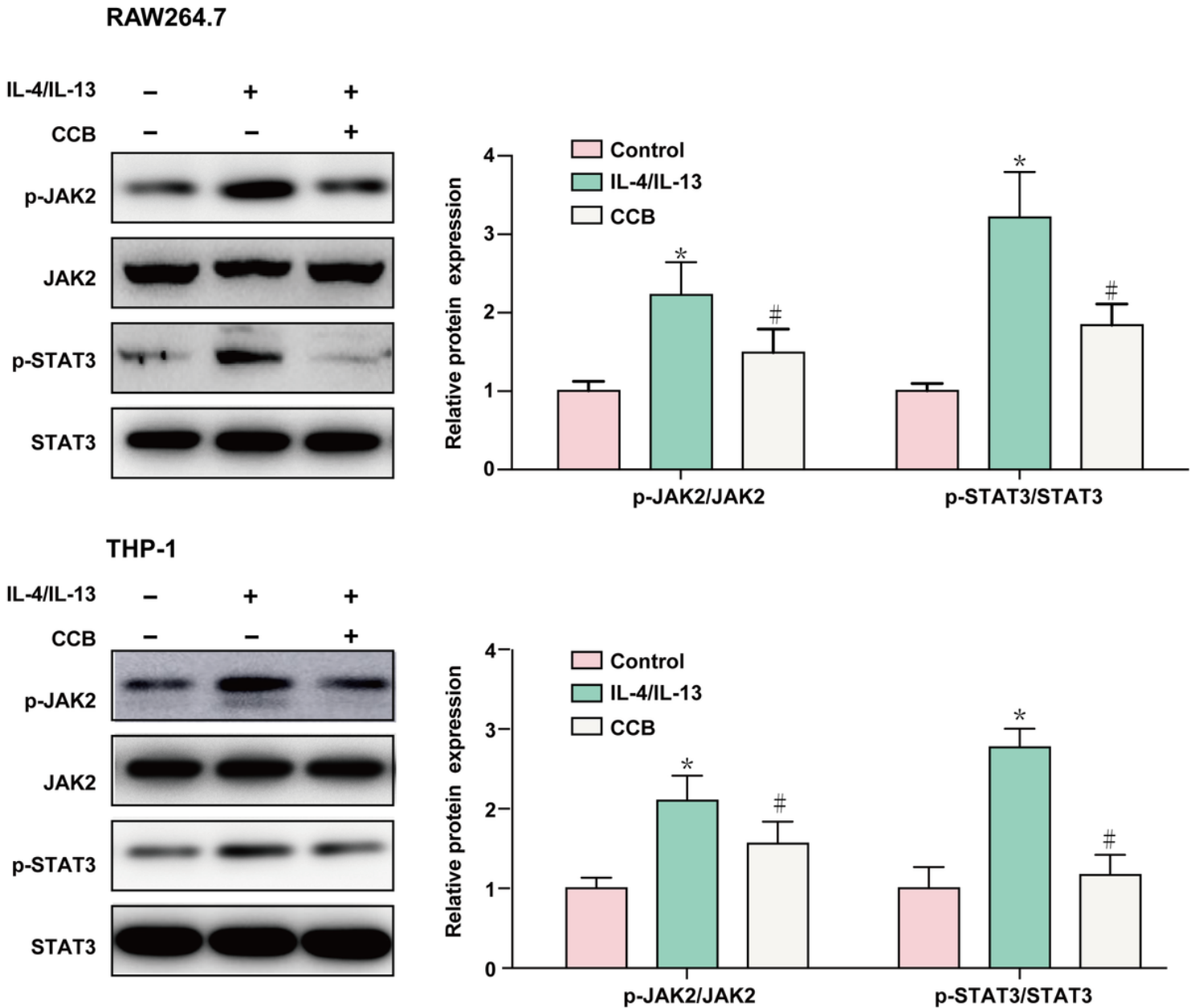


Figure 3

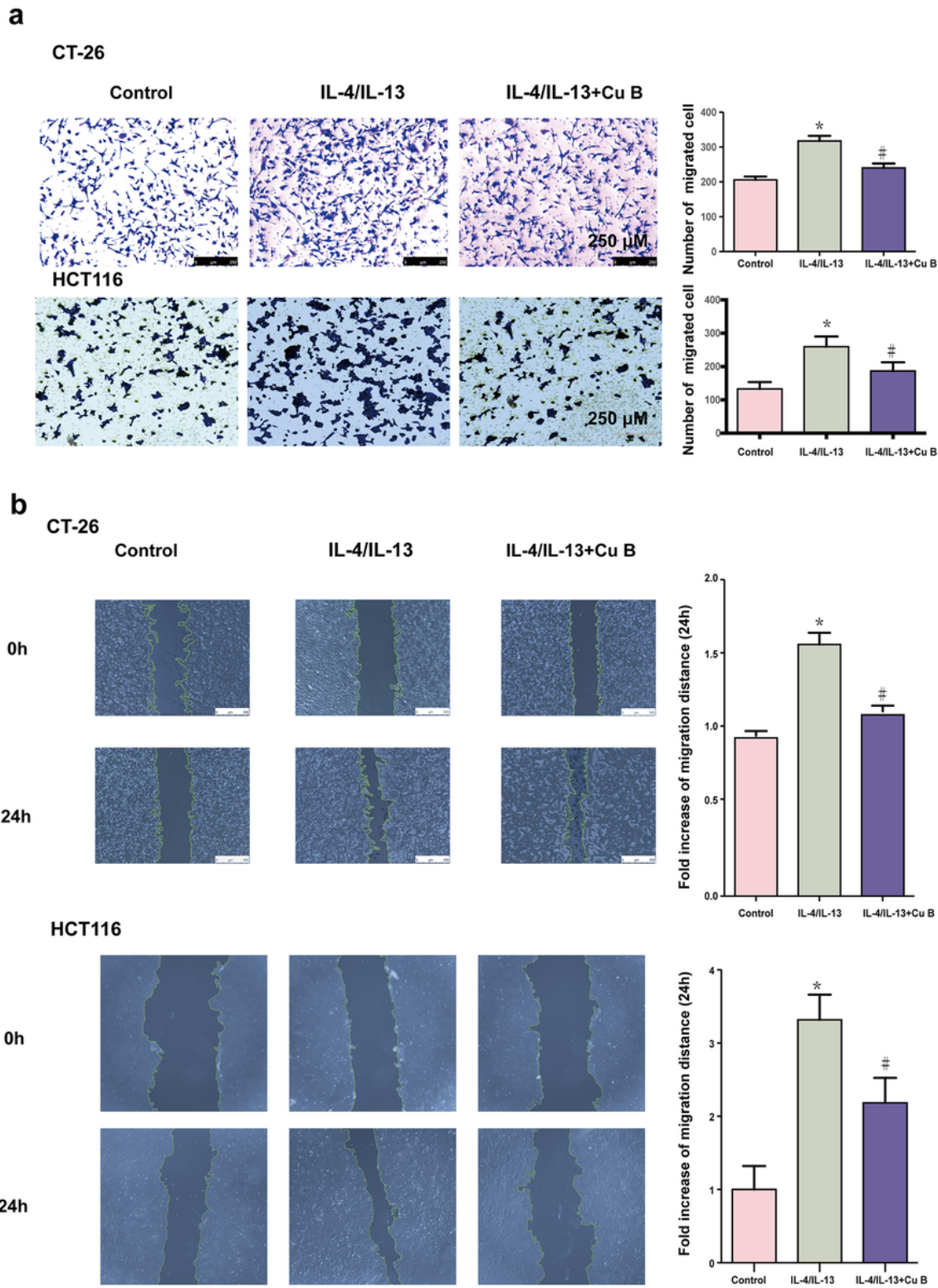
Cucurbitacin B inhibits M2 macrophage polarization (a) FACS (b)RT-PCR analysis of the expression of various cytokines and chemokines in M2 macrophages treated with different cucurbitacin B. (n=4, \*p<0.05 vs control group, #p<0.05 vs IL-4/IL-13-stimulate macrophage group)

**a**



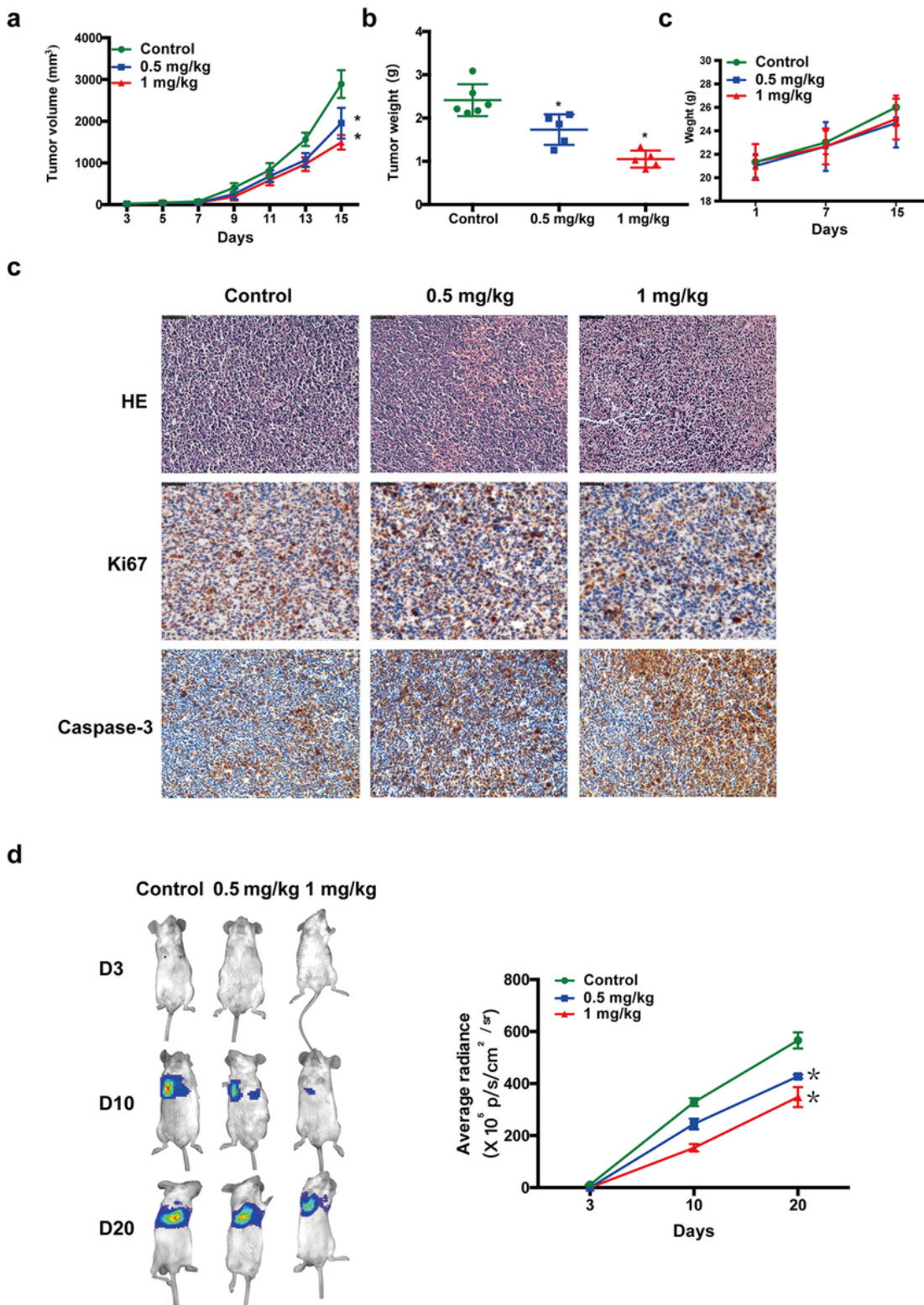
**Figure 4**

Cucurbitacin B suppressed macrophages differentiation via JAK2/STAT3 signaling pathway (a) Western blotting was used detect the expression of p-JAK2, JAK2, p-STAT3 and STAT3 protein in macrophages. (n=4, \*p<0.05 vs control group, #p<0.05 vs IL-4/IL-13-stimulate macrophage group)



**Figure 5**

Cucurbitacin B-stimulated macrophages-colon cancer cell crosstalk inhibited cell migration (a) The CT-26 and HCT116 cells migration activity was assessed by transwell assay after treatment with CCB-stimulated macrophages (b) The CT-26 and HCT116 migration activity was determined using wound healing assay after treatment with CCB-stimulated macrophages (n=4, \*p<0.05 vs control group, #p<0.05 vs IL-4/IL-13-stimulated macrophage group)



**Figure 6**

Cucurbitacin B inhibited tumor growth and CT26-Luc tumor metastasis to the lung in vivo. (a) Xenograft tumor growth curves (b) Tumor weights (c) Body weight (d) Representative images of the immunohistochemical staining of Ki67 and Caspase-3. (e) The tumor load was evaluated via bioluminescence 3, 10 and 20 days after inoculation. (n=6, \*p<0.05 vs control group)

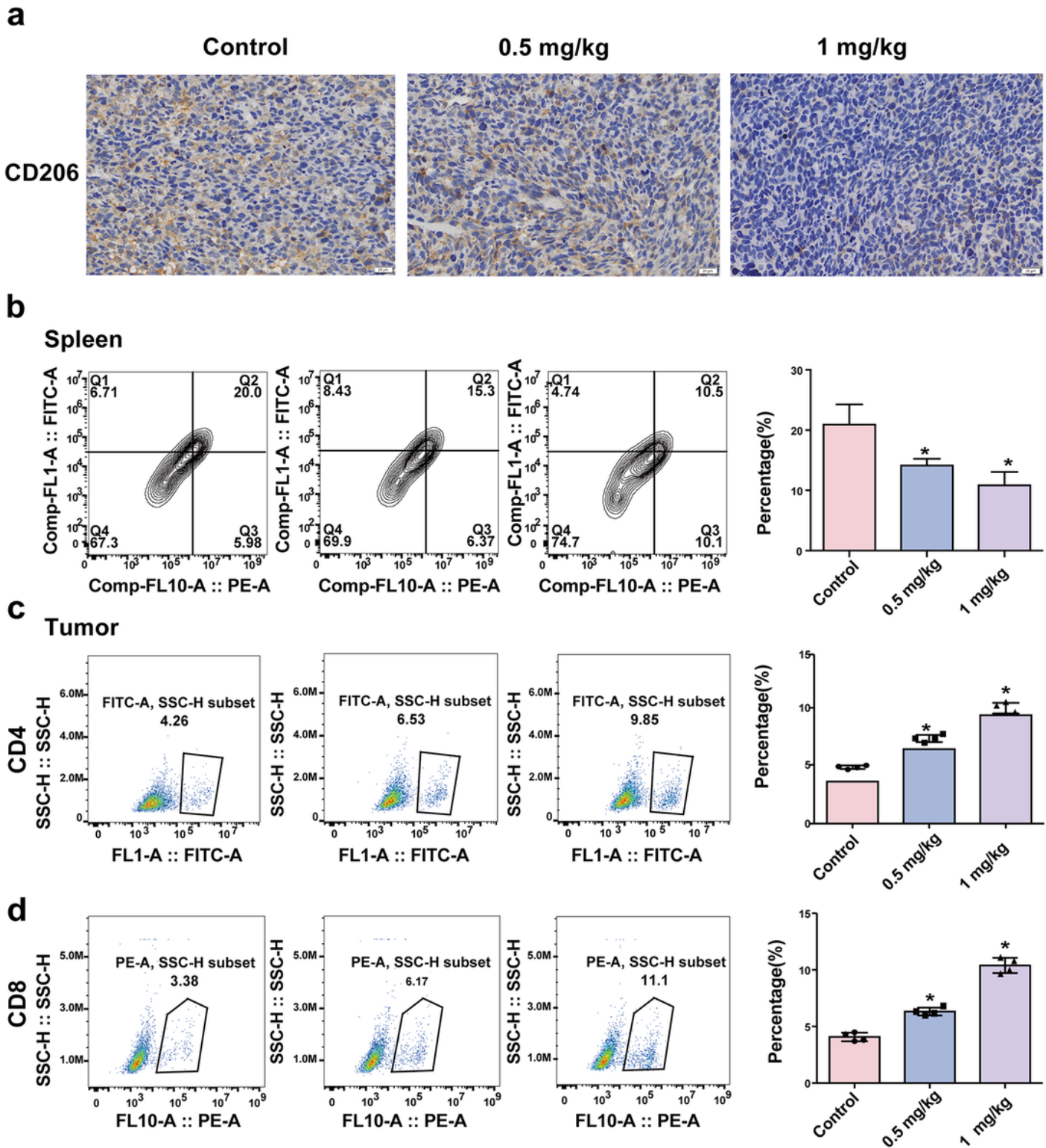


Figure 7

Cucurbitacin B regulated M1 and M2-like macrophage and enhanced T cell infiltration (a) The expression of CD206 in mouse tissue determined by IHC. (b) The expression of CD206/CD11c in mouse spleen determined by Flow cytometry (c-d) Frequencies of CD4+ and CD8+ cells in mouse tumor tissue were measured by Flow cytometry (n=4, \*p<0.05 vs control group)