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Baicalein and baicalin promote antitumor immunity by suppressing PD-L1 expression in hepatocellular carcinoma cells

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ABSTRACT

Blocking the PD-L1/PD-1 pathway to prevent the immune evasion of tumor cells is a powerful approach for treating multiple cancers, including hepatocellular carcinoma (HCC). Previous studies have shown that baicalein and baicalin are directly cytotoxic to some tumors, here we demonstrate that in addition to direct cytotoxicity, these two flavonoids stimulate the T cell mediated immune response against tumors through reduction of PD-L1 expression in cancer cells. Interestingly, more significant tumor regression was observed in BALB/c mice than in BALB/c-nu/nu mice after baicalein and baicalin treatment. PD-L1 upregulation induced by interferon-γ (IFN-γ) was significantly inhibited by these two flavonoids *in vitro*. Both baicalein and baicalin enhanced the cytotoxicity of T cells to eliminate tumor cells, which was abrogated after HCC cells were transfected with a PD-L1 overexpression plasmid or after T cells were pretreated with an anti-PD-1 blocking antibody. Further mechanistic research indicated that the IFN-γ-induced expression and promoter activity of PD-L1 were suppressed by these two flavonoids, and these effects were mediated by STAT3 activity inhibition. Therefore, baicalein and baicalin decreased STAT3 activity, further downregulated IFN-γ-induced PD-L1 expression and subsequently restored T cell sensitivity to kill tumor cells. Our findings provide novel insight into the anticancer effects of baicalein and baicalin through which tumor growth is inhibited by PD-L1 expression downregulation and suggest that these flavonoids have great potential for clinical treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading global causes of cancer-related death [1,2]. Radical treatment, including surgical resection and liver transplantation, can be used to treat HCC, but high recurrence often occurs because most HCC patients are diagnosed at a late stage [3,4]. Therefore, it is of great significance to find an effective therapeutic approach to improve the survival and prognosis of patients with HCC. Emerging evidence suggests that cancer immune suppression and immune escape play essential roles in tumor progression. Immunotherapy, which aims to activate the immune response to recognize and eradicate cancer cells, has emerged as a promising therapy for HCC [5,6]. Among the various immunotherapy approaches, immune checkpoint blockade is the most promising strategy [7,8].

The programmed cell death-ligand (PD-L) 1/programmed cell death protein (PD)-1 pathway has been identified as the most critical immune checkpoint in immunotherapy [9]. PD-L1 is expressed on immune cells and multiple cancer cells, including HCC [10]. The PD-L1 molecule on malignant cells can bind to its receptor PD-1, which is expressed on cytotoxic T lymphocytes (CTLs), and thus inhibits the antitumor function of CTLs and leads to tumor evasion [11]. Studies have reported that PD-L1 upregulation was induced by interferon-γ (IFN-γ) secretion by metastases-infiltrating lymphocytes [12,13]. Therefore, drugs that could downregulate IFN-γ-induced PD-L1 expression in cancer cells and

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eventually restore the function of CTLs are promising for HCC immunotherapy.

Flavonoids are natural polyphenols found in many fruits and vegetables and have many proven beneficial biological functions. Baicalein and its conjugate baicalin are active components of *Scutellaria baicalensis* Georgi. These two components are widely used as traditional Chinese herbal medicines for treating many diseases, including inflammation and infectious diseases [14,15]. In addition, increasing research has indicated that both baicalein and baicalin exhibit strong antitumor effects *via* inducing apoptosis and senescence in cancer cells [14,16]. However, whether baicalein and baicalin could affect the immune response in cancer development has not yet been investigated.

In this study, we investigated the antitumor and immunomodulatory effects of baicalein and baicalin on HCC *in vitro* and *in vivo*. Upon verifying that the antitumor effects of baicalein and baicalin were partly mediated by immune regulation, we found that baicalein and baicalin decreased STAT3 activity and further downregulated IFN-γ-induced PD-L1 expression. As a result, these two drugs restored T cell sensitivity to kill tumor cells. Therefore, our findings revealed a novel inhibitory effect of baicalein and baicalin on HCC, which might have great potential for clinical treatment.

2. Materials and methods

2.1. Cell viability assay

In this study, cell viability was determined with a Cell Counting Kit-8 (CCK-8) assay as previously described [17]. Briefly, the human liver cancer cell lines SMMC-7721 and HepG2 (5×10^3) were seeded in 96-well plates overnight and then treated with different concentrations of baicalein and baicalin (Sigma-Aldrich Co., St. Louis, MO, USA) (ranging from 0 to 80 μM). After 24 h, cell viability was measured by CCK-8 assays (Dojindo, Tokyo, Japan).

2.2. Flow cytometric analysis

Cell surface PD-L1, PD-1 and major histocompatibility class I (MHC I) expression levels were detected by flow cytometry as previously described [18]. Briefly, cells were harvested by centrifugation at 300 ×*g* for 5 min, suspended in PBS/BSA buffer (0.1% BSA in PBS) and then incubated with PE-conjugated mouse IgG2b or PE-conjugated anti-human PD-L1 antibody or FITC-conjugated anti-human PD-1, or FITC-conjugated mouse IgG2b or FITC-conjugated anti-human MHC I antibodies (BioLegend, San Diego, CA, USA) at room temperature for 30 min. Next, the samples were washed three times, resuspended in 500 μL of PBS and analyzed by flow cytometry using an ACEA NovoCyte flow cytometer (San Diego, CA, USA).

2.3. Lymphocyte-conditioned medium preparation

Lymphocyte-conditioned medium was prepared as previously described [18]. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult volunteers after obtaining their informed consent in accordance with protocols approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. PBMCs were separated with Biocoll separating solution (Biochrom AG, Berlin, Germany) and centrifuged at 560 ×*g* for 30 min at room temperature. Then medium unconditioned or conditioned by activated lymphocytes was collected, centrifuged at 2500 ×*g* for 5 min and filtered through 0.22-μm filters to eliminate cell debris. Then, the medium was added to SMMC-7721 or HepG2 cells treated with baicalein or baicalin for an additional 24 h. After incubation, the cells were collected, and surface PD-L1 expression was analyzed by flow cytometry as described above.

2.4. Western blotting analysis

Western blotting was performed according to a previously described method [17]. Briefly, protein was extracted from cells and tumor tissues with RIPA lysate buffer (Beyotime Biotechnology, Shanghai, China). Then, the lysates were separated by SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes, and immunoblotted with anti-PD-L1 (Abcam, Cambridge, MA, USA), anti-STAT3 (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-STAT3 (P-STAT3) (Cell Signaling Technology, Beverly, MA, USA), and anti-GAPDH (Proteintech, Wuhan, China) antibodies.

2.5. Immunofluorescence

SMMC-7721 cells were seeded onto cell culture dishes with glass bottoms (Nest, Shanghai, China). After inoculation, the cells were washed with PBS three times, fixed with 4% paraformaldehyde for 15 min, and then washed with PBS three times. Next, the cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 5% BSA in PBS for 1 h at room temperature. All samples were stained with anti-STAT3 antibodies (Cell Signaling Technology, Beverly, MA, USA) overnight. After removing the antibodies, the samples were washed with PBS three times and incubated with secondary antibodies at room temperature for 30 min. After the samples were washed with PBS three times, the nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Shanghai, China) and observed by confocal microscopy.

2.6. Co-culture experiments and IL-2 measurement

Co-culture experiments were conducted as previously described with modifications [19–22]. Jurkat T cells were infected with control or PD-1 overexpression lentiviruses. Cell surface PD-1 expression was confirmed by FACS analysis (Supplementary Fig. 1). Jurkat T cells infected with PD-1 overexpression lentiviruses were activated with 100 ng/mL CD3 antibody (BioLegend, San Diego, CA, USA) and 100 ng/mL CD28 antibody (BioLegend, San Diego, CA, USA) before co-culture with cancer cells for 72 h. HepG2 cells and SMMC-7721 cells were pretreated with DMSO, 10 μM baicalein, or 40 μM baicalin for 4 h and then cultured in the presence of 10 ng/mL IFN-γ (R&D Systems, Minneapolis, MN, USA) for 24 h. Then, the drugs were removed, and the cells were collected, washed, resuspended and distributed into 96-well plates with an effector T cell to target ratio of 10:1 for 24 h. The cytotoxic activity of the target cells was measured with a cytotoxicity LDH assay kit (Dojindo, Japan) according to the manufacturer's instructions. The supernatants were collected, and IL-2 in the medium was measured using an IL-2 ELISA kit (R&D Systems, Minneapolis, MN, USA).

CTL cytotoxicity was determined as previously described [18]. Human CD8-positive T cells were enriched from healthy donor blood using RosetteSep™ Human $CD8^+$ T Cell Enrichment Cocktail (StemCell, Vancouver, BC, Canada), and the cells were then stimulated with ImmunoCult Human CD3/CD28 T Cell Activator Cocktail (Vancouver, BC, Canada) for 72 h. Then, the cytotoxic activity of CTLs toward the target tumor cells was measured as described above, the difference is that the time of co-incubation is 6 h.

In the blocking assay, blockade of PD-1 on $CD8^+$ T cells were performed as previously described [21]. $CD8^+$ T cells were pretreated with 2 mg/mL anti-human PD-1 antibody or 2 mg/mL normal goat IgG control antibody as isotype control group (R&D Systems, Minneapolis, MN, USA) for 4 h. Next, the cytotoxic activity of CTLs toward the target tumor cells was measured as described above.

2.7. Overexpression plasmid transfection

HCC cells were plated and allowed to attach overnight. Then, the cells were transfected with pcDNA3, pGMSTAT3, pGV219 vector or

pGV219PD-L1 using Lipofectamine 2000 (Invitrogen, MA, USA) for 48 h according to the manufacturer's protocol.

2.8. PD-L1 promoter luciferase assays

Luciferase assays were conducted as previously described [23]. Briefly, SMMC-7721 and HepG2 cells were transiently transfected with the STAT3 overexpression plasmid or vector control. Then, the cells were further transfected with the pGL3-PD-L1 plasmid (GenePharma, Shanghai, China). The Renilla luciferase plasmid pRL-TK served as an internal control. One day later, baicalein, baicalin, and IFN-γ were added. After treatment, the lysates were prepared using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions. Bioluminescent firefly and Renilla activities were measured using a Centro XS3 LB 960 Microplate Luminometer (Berthold Technologies).

2.9. In vivo experiments

An H22-cell-bearing mouse model was established as previously described [24]. Eight-week-old male BALB/c mice or four-week-old BALB/c-nu/nu mice were injected subcutaneously with 4×10^6 H22 cells in 100 μL of PBS. 24 h after tumor implantation, the mice received intraperitoneal drug injections according to the different experimental schemes. In the baicalein and baicalin individual treatment experiment, the mice were divided into three groups and administered the following daily: control group (PBS), baicalein-treated group (50 mg/kg body weight) and baicalin-treated group (80 mg/kg body weight). The mice received these injections for 12 days. All procedures involving animals and their care in this study were performed in strict accordance with protocols approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

During treatment, the tumor volume (TV) of each mouse was measured every three days and calculated using the following formula: $TV = 1/2 \times a \times b^2$, where a and b are the longest and shortest diameters of the tumors in each mouse, respectively.

All of the mice were sacrificed by cervical dislocation 24 h after the final treatment. The tumors from each mouse were harvested, weighed, and then embedded in paraffin for immunohistochemistry staining.

2.10. Statistical analysis

The data are shown as the means ± standard deviation (SD) and were analyzed using SPSS 16.0 software. Statistical significance of the differences between the control and treatment groups was analyzed by two-tailed unpaired *t*-test. P-values of < 0.05 were considered statistically significant.

3. Results

3.1. Baicalein and baicalin inhibit tumor growth and immunosuppression in vivo

To investigate the effect of baicalein and baicalin on antitumor immunity, we compared the antitumor effects of these two flavonoids in immunocompetent BALB/c mice and immunodeficient BALB/c-nu/nu mice using a H22 liver cancer model.

As shown in Fig. 1A–G, baicalein and baicalin treatment evidently suppressed tumor growth in H22-bearing BALB/c mice and BALB/c-nu/nu mice. However, the tumor regression effect was more obvious in BALB/c mice than in BALB/c-nu/nu mice after baicalein and baicalin treatment. The tumor inhibitory rates were 78% and 80% in the baicalein- and baicalin-treated BALB/c mice groups, respectively. In contrast, the tumor inhibitory rates were both 60% in the baicalein- and baicalin-treated BALB/c-nu/nu mice groups. These data suggest that the antitumor effects of baicalein and baicalin are closely related to the immune response. Then, the effects of baicalein and baicalin on the tumor microenvironment were further investigated. The immunohistochemistry analyses of resected tumor tissues from BALB/c mice showed that the numbers of $CD8^+$ cells were apparently higher in the tumor tissues from baicalein- or baicalin-treated mice than in those from the control mice (Fig. 1H). These findings indicated that these two drugs could stimulate the immune response. Moreover, PD-L1 expression, one of the important inhibitory immune checkpoint regulators, was significantly downregulated in the tumor tissues of the baicalein and baicalin treatment groups (Fig. 1I). Similar results in the western blotting of tumor tissues showed that PD-L1 expression was reduced in baicalein- or baicalin treated group (Supplementary Fig. 2A and B). Altogether, these data indicated that baicalein and baicalin increase the activity of T cells, which may contribute to their antitumor effects.

3.2. Baicalein and baicalin significantly inhibit IFN-γ-induced PD-L1 expression in HCC cells

It has been reported that both baicalein and baicalin can inhibit tumor growth in various cancers [14,16,25]. Therefore, to test the antiproliferative effect of baicalein and baicalin on liver cancer cells, two human liver cancer cell lines (SMMC-7721 and HepG2) were used, and tumor cell viability was determined using a CCK-8 assay. The results showed that both baicalein and baicalin strongly inhibited SMMC-7721 and HepG2 cell proliferation in a dose-dependent manner (Supplementary Fig. 3A–D). Then we selected concentrations near the IC_{50}, which is commonly used to study the antitumor immunotherapeutic activity of cytotoxic drugs [19,26], to investigate both the immunomodulatory effect of baicalein (10 μM) and baicalin (40 μM) on HCC cells *in vitro*.

It has been reported that IFN-γ, which is secreted by activated T cells, could stimulate PD-L1 expression [12,13]; thus, we measured the effects of baicalein and baicalin on IFN-γ-induced PD-L1 expression in both SMMC-7721 and HepG2 cells. The flow cytometry results showed that IFN-γ treatment strongly stimulated PD-L1 surface expression, while both baicalein and baicalin remarkably attenuated PD-L1 upregulation (Fig. 2A–D). Additionally, the results of treating SMMC-7721 cells with different doses of baicalein and baicalin indicated that both compounds dose-dependently inhibited IFN-γ-induced PD-L1 expression (Supplementary Fig. 4A–D). Similar results were obtained using conditioned medium of human activated lymphocyte (CM)-treated cancer cells; baicalein and baicalin decreased the membrane surface expression of PD-L1 induced by CM (Fig. 2E–H). In accordance with the flow cytometry data, the western blotting assay results indicated that baicalein and baicalin treatment substantially reduced the IFN-γ-induced PD-L1 upregulation (Fig. 2I–L). In addition to IFN-γ-induced PD-L1 expression, we detected constitutive PD-L1 expression after baicalein and baicalin treatment. The data showed that baicalein and baicalin slightly suppressed constitutive PD-L1 expression (Supplementary Fig. 5).

In addition to PD-L1, IFN-γ could also induce MHC I molecules, which could stimulate T cell activity. Because both baicalein and baicalin treatment impaired the IFN-γ-induced PD-L1 upregulation, we next investigated whether MHC I molecules were inhibited by these two drugs in the same process. The data obtained indicated that MHC I molecule levels were not lower in baicalein- and baicalin-treated cells than in IFN-γ-treated cells (Fig. 2M–P). Therefore, baicalein and baicalin impaired only IFN-γ-induced PD-L1 expression and had no effects on MHC I molecules.

All of the results mentioned above indicated that both baicalein and baicalin could inhibit IFN-γ-induced PD-L1 upregulation in HCC cells *in vitro*.

3.3. Baicalein and baicalin enhance T cell-mediated cancer cell death

Because PD-L1 is crucial in mediating the immune evasion of cancer

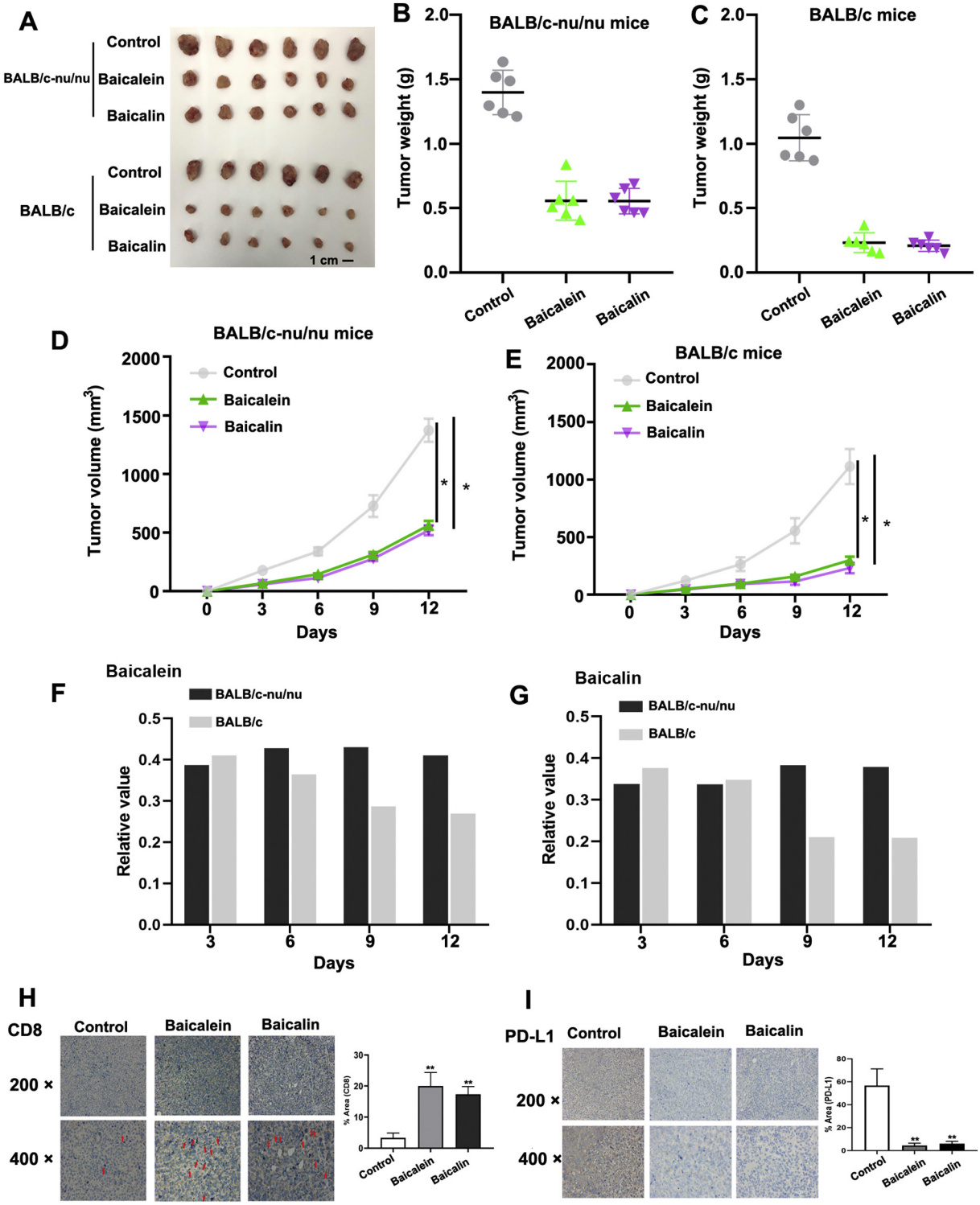

Fig. 1. Baicalein and baicalin inhibited H22 tumor growth and stimulated the host T cell response. H22 cells were inoculated subcutaneously into BALB/c-nu/nu mice or BALB/c mice, and the mice were divided randomly into 3 groups: control, baicalein or baicalin; the mice received daily intraperitoneal injections for 12 consecutive days. (A) Photograph of tumor tissues from each group. (B, C) The weights of resected tumors in were measured and plotted. (D, E) Tumor volumes in each group were measured every other day during the treatment. (F, G) Comparison of tumor progression in baicalein or baicalin-treated animals relative to untreated animals. (H, I) Resected tumor tissue sections from BALB/c mice were analyzed by immunohistochemistry using anti-CD8 (H), and anti-PD-L1antibodies (I). Representative micrographs are shown (200×, 400×). Each group contained 6 mice; the data represent the means ± SD; *P < 0.05, **P < 0.01, compared with the control group.

cells, we next investigated whether baicalein and baicalin could affect T cell-mediated HCC cell killing. In this experiment, we generated PD-1-expressing Jurkat T cells. Cultured SMMC-7721 cells and HepG2 cells were pretreated with IFN-γ alone or with baicalein or baicalin, and the drugs were then removed before co-culture with PD-1-expressing Jurkat T cells. As Fig. 3A and B shows, Jurkat T cell-mediated HCC cell killing activity was significantly inhibited in IFN-γ-treated cancer cells. However, this inhibitory effect was reversed by baicalein and baicalin.

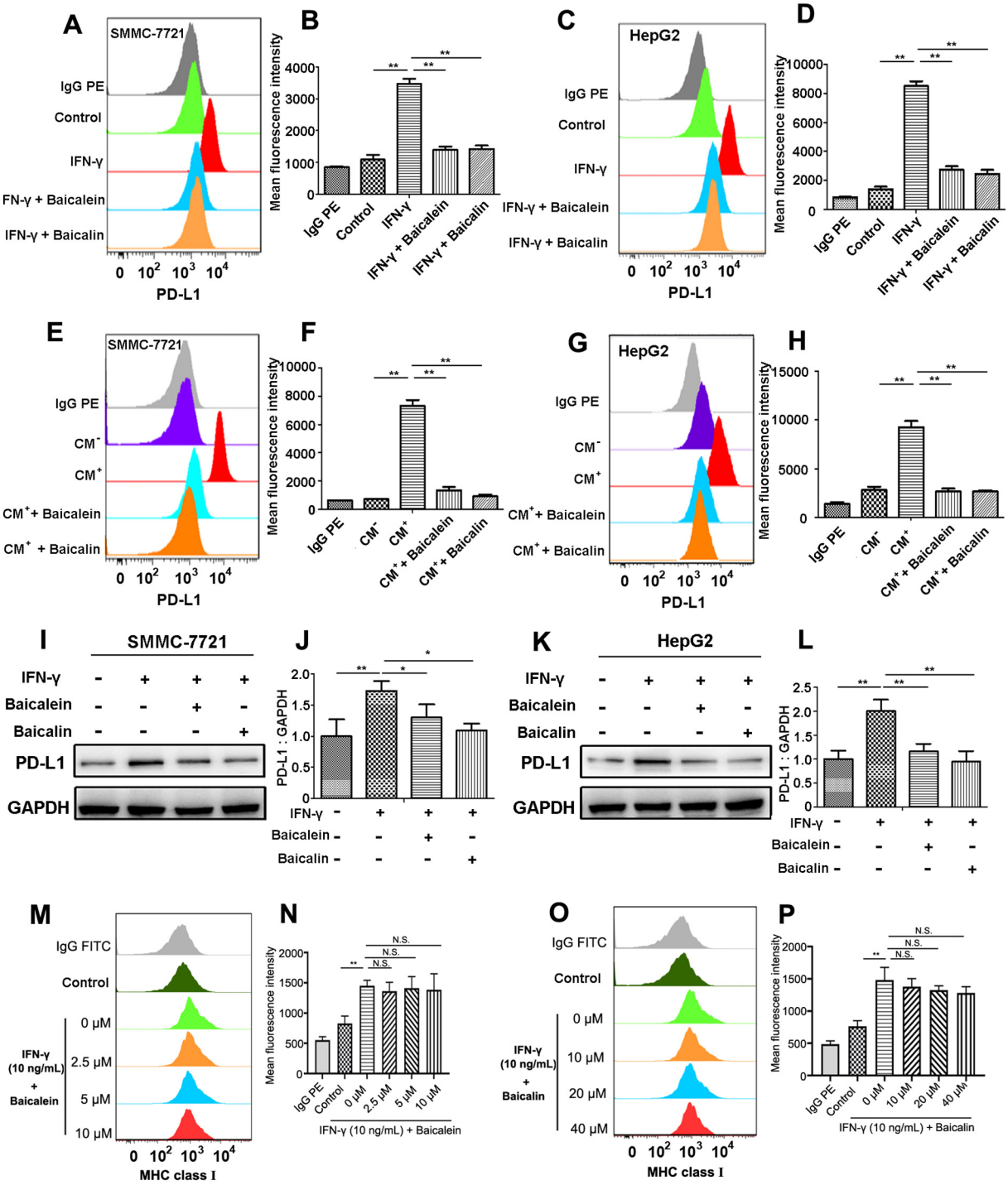

Fig. 2. Baicalein and baicalin suppress IFN-γ-induced PD-L1 expression in human HCC cells. (A–D) HCC cells were pretreated with DMSO, baicalein (10 μM), or baicalin (40 μM) for 4 h and then treated with IFN-γ (10 ng/ml) for 24 h. The cells were harvested, and membrane PD-L1 expression was determined by flow cytometry. (E–H) HCC cells were treated with baicalein (10 μM), baicalin (40 μM) or conditioned medium from activated (CM^+) or nonactivated (CM^-) lymphocytes for 24 h. Then, surface PD-L1 expression was determined by flow cytometry. (I–L) HCC cells were treated as described in A–D, after which the cells were lysed, and PD-L1 expression was detected by western blotting. GAPDH was used as a loading control. The column charts below show quantitation data for relative PD-L1 expression. (M–P) Baicalein and baicalin had no effects on the expression of MHC I molecules. SMMC-7721 cells were pretreated with DMSO, baicalein (2.5, 5, or 10 μM), or baicalin (10, 20, or 40 μM) for 4 h and then with IFN-γ (10 ng/ml) for 24 h. The cells were harvested, and MHC I molecule expression was determined by flow cytometry. The histograms shown in panels A, C, E, G, M and O are representative of triplicates, and the combined results are presented in panels B, D, F, H, N and P. The data are presented as the mean ± SD from three independent experiments. *P < 0.05 and **P < 0.01, compared with the IFN-γ- or CM-treated group.

Furthermore, because IL-2 is an indicator of T cell activity, IL-2 secretion in the media of HCC cells and Jurkat T cell co-cultures was also detected by ELISA. As Fig. 3C and D illustrates, the IL-2 level was remarkably decreased in the IFN-γ-treated group, indicating that T cell activity was compromised. However, in the baicalein- and baicalin-treated groups, the IL-2 levels in the medium were strongly restored, suggesting the positive effects of these two drugs on T cell activity. Additionally, $CD8^+$ T cells were adopted to evaluate CTL cytotoxicity.

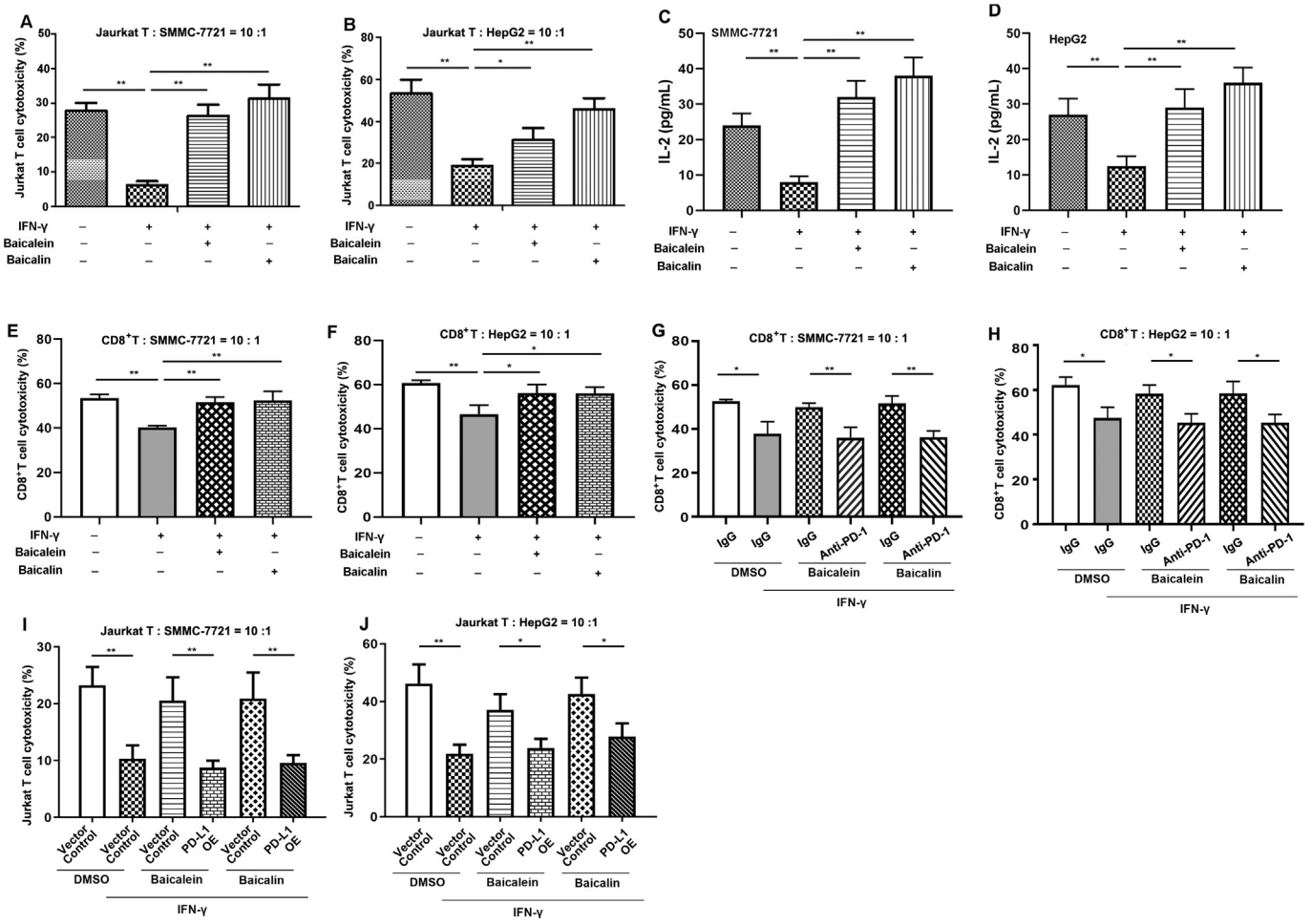

Fig. 3. Baicalein and baicalin enhance T cell-mediated cell death by downregulating PD-L1. (A–F) HCC cells were treated with DMSO, baicalein (10 μM) or baicalin (40 μM) for 4 h and then with IFN-γ (10 ng/ml) for 24 h. Next, the drugs were removed, and the cells were co-cultured with PD-1-overexpressing Jurkat T cells with effector to target ratios of 10:1. After 48 h of incubation, cytotoxicity was analyzed (A, B), and IL-2 secretion in the media was measured (C, D). HCC cells were treated as described above and then cultured with activated $CD8^+$ T cells with an effector to target ratio of 10:1. After 6 h of incubation, cytotoxicity was analyzed (E, F). (G, H) Blocking PD-1 impaired baicalein- and baicalin-induced T cell-mediated killing. Activated CD8+ T cells were pretreated with an anti-PD-1 blocking antibody. Then, the cells were co-cultured with IFN-γ and baicalein- and baicalin treated HCC cells, and CTL activity detection assays were performed as previously described. (I, J) PD-L1 overexpression attenuated the increased T cell-mediated killing sensitivity induced by baicalein and baicalin. HCC cells were transfected with a PD-L1 overexpression plasmid or vector control. HCC cells were treated, and a Jurkat T cell-mediated killing assay was performed as described above. The data are presented as the mean ± SD of three independent experiments. *P < 0.05 and **P < 0.01, compared with the IFN-γ-treated cells.

Similar results were presented in Fig. 3E and F. The cytotoxicity of CD8+ T cells was downregulated in IFN-γ-treated HCC cells, and this reduction was inhibited by baicalein and baicalin.

To determine the potential role of PD-L1 and PD-1 in baicalein- and baicalin-induced CTL-mediated lysis, PD-L1 overexpression was induced in HCC cells by plasmid transfection, and an anti-PD-1 antibody was used. The results are shown in Fig. 3G and H, IFN-γ suppressed CTLs cytotoxicity, and this reduction was rescued by baicalein and baicalin treatment. However, baicalein- and baicalin-induced CTL-mediated lysis was inhibited in CTLs pretreated with a PD-1 blocking antibody. Moreover, Fig. 3I and J also indicated that IFN-γ inhibited the Jurkat T cells mediated tumor killing activity (groups 1 and 2) and baicalein and baicalin could reverse this inhibitory effect (groups 3 and 5). When HCC cells were transfected with the PD-L1 overexpression plasmid, these two flavonoids-induced T cell activities were abrogated (groups 3 and 4, groups 5 and 6). On the basis of our results, we propose that baicalein- and baicalin-mediated enhancement of T cell-mediated lysis in HCC cells is a result of increased interaction between PD-L1 on tumor cells and PD-1 on CTLs.

3.4. Baicalein- and baicalin-mediated PD-L1 expression downregulation is mediated by STAT3 activation

It has been well established that activated STAT3 is involved in IFN-γ-induced PD-L1 expression [27,28]. In addition, it has been reported that baicalein and baicalin could inhibit STAT3 phosphorylation [29,30]. Hence, we speculated that these two drugs attenuate PD-L1 expression by inhibiting STAT3. Western blotting analysis indicated that IFN-γ stimulated STAT3 phosphorylation, which was impaired by baicalein and baicalin (Fig. 4A–D). Similar results was obtained in the western blotting of H22-bearing BALB/c mice resected tumor tissues and showed that STAT3 phosphorylation expression was also down-regulated in baicalein- or baicalin treated group (Supplementary Fig. 2A and C). Moreover, confocal microscopy further confirmed that both baicalein and baicalin inhibited IFN-γ-induced STAT3 nuclear accumulation (Fig. 4E). Therefore, we can preliminarily conclude that baicalein and baicalin suppress IFN-γ-induced PD-L1, at least in part, by inhibiting STAT3 activation.

Previous studies [31–33] and our experiments verified that STAT3 overexpression can simultaneously increase STAT3 and phosphorylated STAT3 levels (Supplementary Fig. 6). Therefore, to reveal the role of

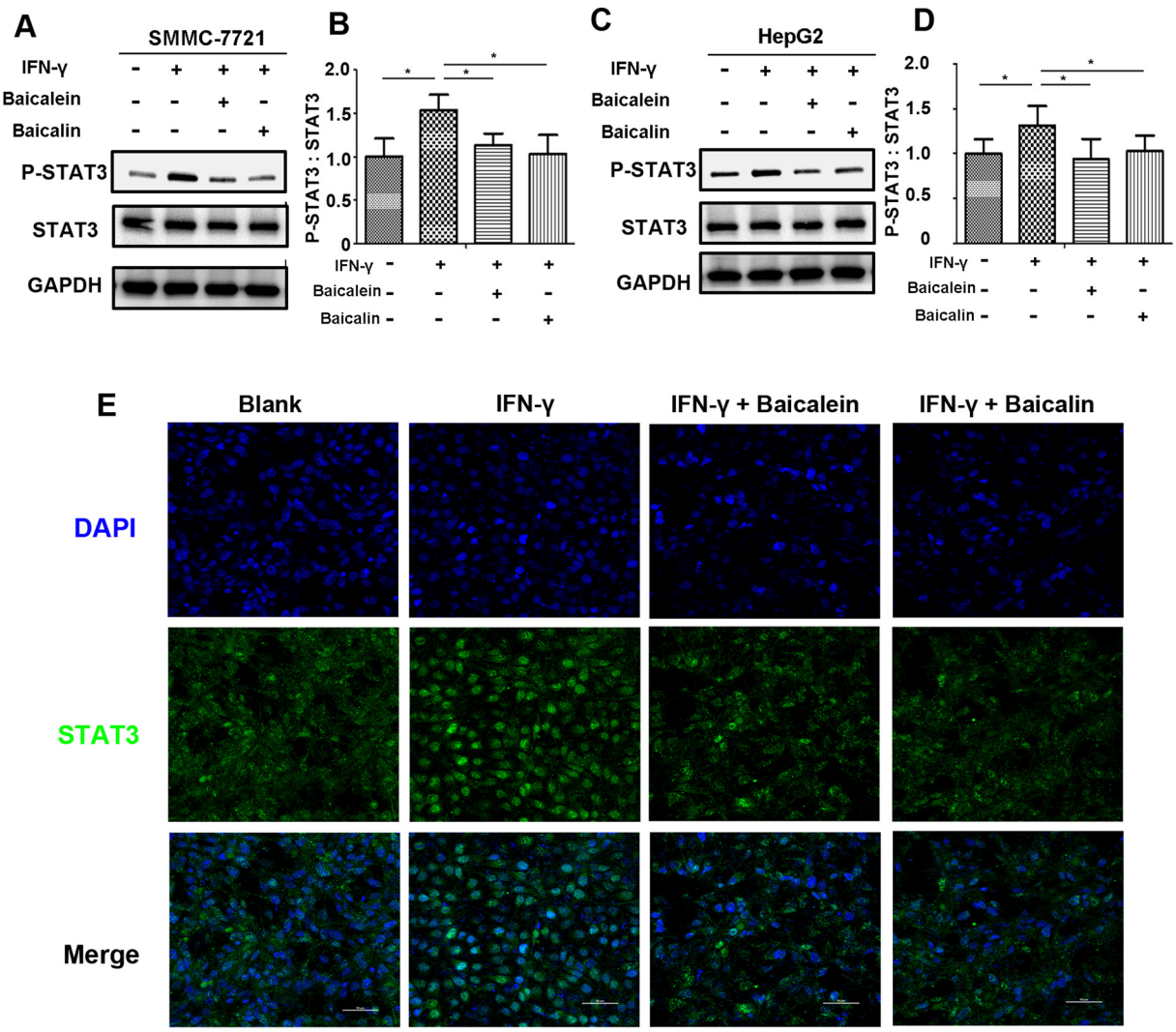

Fig. 4. Baicalein and baicalin attenuate IFN-γ-induced STAT3 phosphorylation in human melanoma cells. HCC cells were incubated with DMSO (blank), baicalein (10 μM) or baicalin (40 μM) for 4 h and then treated with IFN-γ (10 ng/ml) for 24 h. (A–D) Total STAT3 and phosphorylated STAT3 (P-STAT3) levels were detected by western blotting analysis. GAPDH was used as an internal control. (E) STAT3 expression in SMMC-7721 cells was determined by immunofluorescence staining. The data are presented as the mean ± SD from three independent experiments, *P < 0.05, compared with the IFN-γ-treated cells.

STAT3 in baicalein- and baicalin-mediated PD-L1 expression inhibition, STAT3 was overexpressed in cancer cells through transfecting an overexpression plasmid, and the effects were evaluated. The results shown in Fig. 5A–H indicate that IFN-γ induced the PD-L1 expression (groups 1 and 2) and this circumstance was inhibited by baicalein and baicalin (groups 2 and 3). STAT3 overexpression overcame baicalein- and baicalin-induced PD-L1 suppression in both SMMC-7721 and HepG2 cells (groups 3 and 4). Moreover, the luciferase reporter assay results indicated that IFN-γ enhanced the promoter activity of PD-L1, whereas this stimulatory effect was attenuated by baicalein and baicalin (Fig. 5I and J). However, STAT3 overexpression reversed the inhibitory effect of PD-L1 promoter activity induced by baicalein and baicalin. These data indicate that both baicalein and baicalin suppressed PD-L1 expression by activating STAT3.

4. Discussion

Cancer cells exist in a complex environment. Cancer cells interact with host immune cells to promote or inhibit the development of cancer. Currently, immunotherapy is popular in the field of cancer and has resulted in major breakthroughs in treating various cancers. Increasing evidence suggests that blocking immune checkpoints is the most promising approach in immunotherapy. Among the various inhibitory checkpoints, the PD-1/PD-L1 axis has become one of the most crucial pathways of the decade [34]. In preclinical and clinical HCC studies, blockade of the PD-L1/PD-1 pathway has achieved great success. A clinical trial of nivolumab, a PD-1 inhibitor, shows a dramatic tumor size response and a decrease in alfa-fetoprotein in patients with advanced HCC. This success highlights the vital role of PD-L1 in cancer immune evasion and reveals the potential of nivolumab for the treatment of advanced HCC [35]. Moreover, the FDA officially approved nivolumab (Opdivo) to treat HCC patients after sorafenib resistance therapy in 2017 [36], indicating that drugs which could inhibit the PD-1/PD-L1 axis would have great prospective application in treating HCC.

Baicalein and baicalin are flavonoids extracted from Scutellaria root with prominent biological activities, including anti-oxidation, anticancer and anti-inflammation, with little toxicity in normal tissues [15,37,38]. Similar results were obtained in previous studies, and our observations indicated that baicalein, along with baicalin, exhibited strong anti-proliferative activity toward HCC cells. Although the various activities of baicalein and baicalin have previously been reported, whether baicalein and baicalin inhibit tumor growth by regulating the immune system has not yet been reported. In this study, we found that more significant tumor suppression was observed in immunocompetent

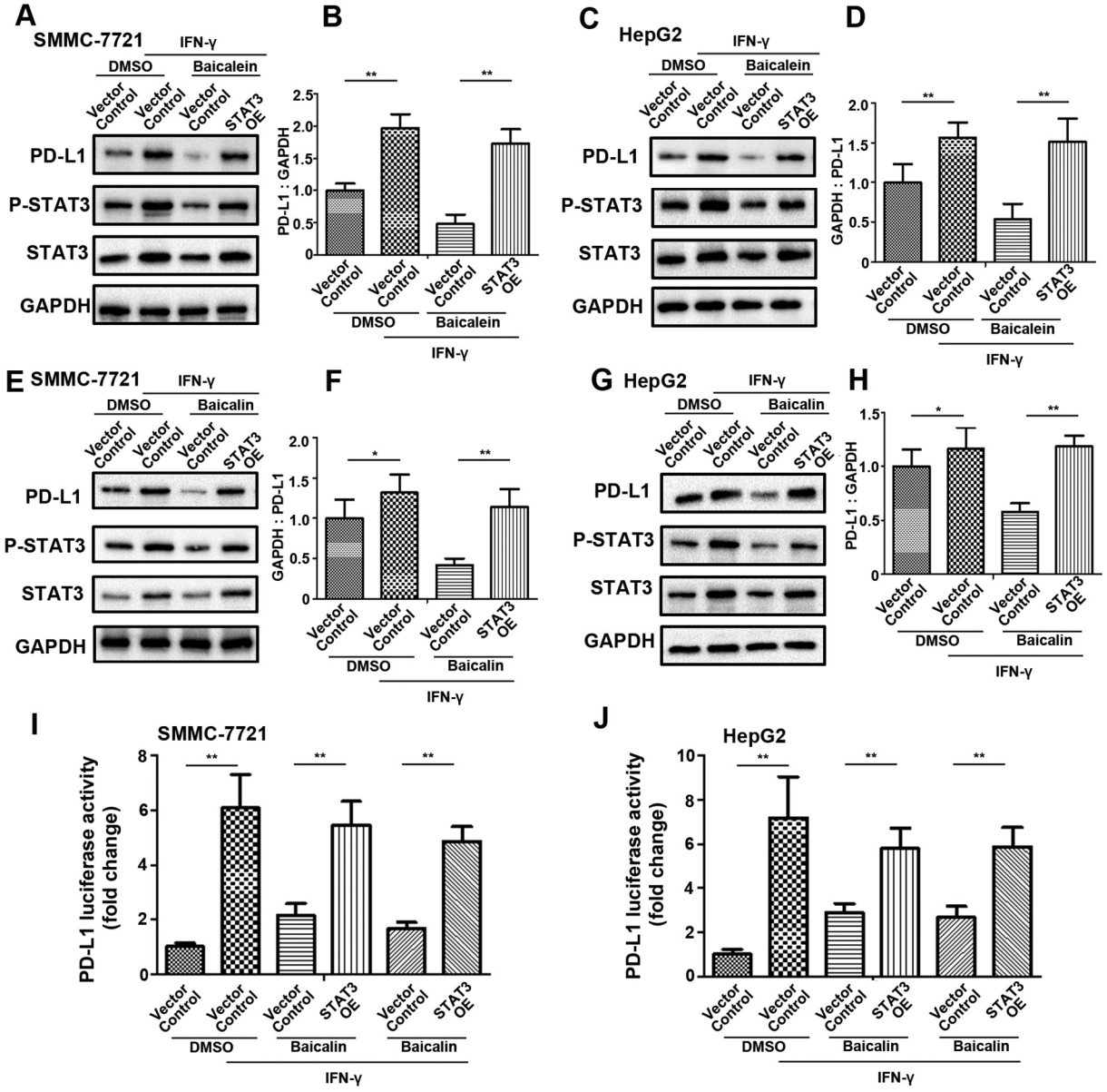

Fig. 5. Baicalein and baicalin inhibition of IFN-γ-induced PD-L1 expression is mediated by STAT3. (A–H) SMMC-7721 and HepG2 cells were transfected with the STAT3 overexpression plasmid or vector control, and the cells were treated with DMSO, baicalein (10 μM) or baicalin (40 μM) for 4 h and then with IFN-γ (10 ng/ml). After 24 h, the cells were lysed and detected by western blotting assay. (I–J) SMMC-7721 cells and HepG2 cells were transfected with the STAT3 overexpression plasmid or vector control. Then, the cells were further transfected with the pGL3-PD-L1 plasmid. The Renilla luciferase plasmid pRL-TK served as an internal control. One day after transfection, baicalein, baicalin, and IFN-γ were added to the medium. Then, the activity of PD-L1 was detected by the Dual-Luciferase Reporter Assay. The data are presented as the mean ± SD from three independent experiments. P-values were calculated using a two-sided unpaired *t*-test, and *P < 0.05, **P < 0.01.

BALB/c mice than in immunodeficient BALB/c-nu/nu mice after baicalein and baicalin treatment, illustrating their immunoregulatory activities.

In the tumor microenvironment, cancer cells escape immune surveillance through various mechanisms, including upregulating immune checkpoints, such as PD-L1. In the H22-bearing BALB/c mouse model, enhanced $CD8^+$ T cell infiltration and PD-L1 downregulation were apparent in the tumor tissues from the baicalein- and baicalin-treated groups. Moreover, it has been reported that IFN-γ produced by lymphocytes in the tumor microenvironment can induce PD-L1 [12,13]. Interestingly, we observed a significant reduction of the effects of baicalein and baicalin on PD-L1 expression levels in HCC cells following IFN-γ induction *in vitro*. Previous studies have shown that the PD-L1/PD-1 pathway mediates immune suppression and cancer evasion through inhibiting T cell cytotoxicity [39]. Further cytotoxicity assays indicated that both Jurkat T cell- and CTL-mediated HCC cell killing activity was inhibited in IFN-γ-pretreated cancer cells, and this reduction was restored by baicalein and baicalin treatment; these effects were attenuated by PD-L1 overexpression in cancer cells and anti-PD-1 blocking. Therefore, our observations revealed that a novel mechanism through which baicalein and baicalin suppress HCC development was potently mediated by suppressing PD-L1 expression and thus enhancing host immunity.

PD-L1 expression is regulated by various signaling pathways. Among these mechanisms, the role of JAK/STATs in PD-L1 regulation has been highlighted [39]. Of the STAT family, both STAT1 and STAT3 can promote PD-L1 transcription after exposure to IFN-γ [18,40,41]. More specifically, activated STAT3 was shown to bind to the PD-L1

promoter and subsequently promote PD-L1 transcription [27,28]. Moreover, baicalein and baicalin have been reported to inhibit the activity of STAT3 [29,30]. Therefore, we suspect that STAT3 is involved in baicalein- and baicalin-mediated reductions in PD-L1 expression. Further results revealed that IFN-γ could activate STAT3 in HCC cells and that this stimulation was suppressed by both baicalein and baicalin. Moreover, when cancer cells were transfected with the STAT3 overexpression plasmid, the baicalein- and baicalin-mediated reductions in PD-L1 expression and promoter activity were impaired, suggesting that baicalein- or baicalin-induced PD-L1 expression downregulation is mediated by inhibiting STAT3 activity. In the present study, we also found that IFN-γ-induced MHC-I upregulation was not reduced by baicalein and baicalin. There are no direct studies prove that STAT3 was involved in IFN-γ-induced MHC-I expression until now. Researches have been reported that some factors activated STAT3 and induce PD-L1, but inhibited MHC I expression simultaneously [42]. It has also been reported that when STAT3 activation was associated with MHC I upregulation [43]. Therefore, the role of STAT3 in mediating MHC I expression is uncertain. Because cell regulation is a complex network system and there are many vital factors involved in IFN-γ regulating MHC I expression [44]. It is possible these factors are not affected by baicalein and baicalin, result in these two drugs suppressing PD-L1 expression while not altering the MHC I expression.

In this study, we demonstrated that the flavonoids baicalein and baicalin strongly inhibited IFN-γ-induced STAT3 activation, leading to PD-L1 reductions in liver cancer cells, which rendered the tumor cells more sensitive to CTLs. The results of this study provide evidence of the anticancer effects of flavonoids with immune-regulatory potential for the clinical treatment of cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105824.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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