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ORIGINAL ARTICLE

Scutellaria extract and wogonin inhibit tumor-mediated induction of T_{reg} cells via inhibition of TGF-β1 activity

Sagar Dandawate · Linford Williams · Nirmal Joshee · Agnes M. Rimando · Sandeep Mittal · Archana Thakur · Lawrence G. Lum · Prahlad Parajuli

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Abstract A number of studies have implicated tumor-induced T_{reg} cell activity in the sub-optimal response to therapeutic vaccines. Development of neo-adjuvant strategies targeting T_{reg} cells is therefore imperative. Scutellaria extracts or constituent flavonoids have shown encouraging efficacy against various tumors, including gliomas, in both pre-clinical and clinical studies. We report here, for the first time, that *Scutellaria ocmulgee* leaf extract (SocL) and flavonoid wogonin could inhibit TGF-β1-induced T_{reg} activity in malignant gliomas. F344 rats, subcutaneously transplanted with F98 gliomas, were treated with SocL. There was a significant inhibition of intra-tumoral TGF-β1 and T_{reg} cell frequency as well as peripheral blood TGF-β1 levels in SocL-treated animals compared to the controls. SocL extract and wogonin also inhibited glioma-induced, TGF-β1-mediated T_{reg} activity in vitro. SocL extract and wogonin also inhibited the secretion of IL-10 in T_{reg} culture; whereas the level of IL-2 was either unchanged or marginally enhanced. We also observed an inhibition of Smad-3, GSK-3β and ERK1/2 signaling by SocL and wogonin in T_{reg} cells, while phosphorylation of P38 MAPK was considerably enhanced, indicating that SocL or wogonin could inhibit the T cells' response to TGF-β1 via modulation of both Smad and non-Smad signaling pathways. Overall, this study suggests that Scutellaria can potentially reverse tumor-mediated immune suppression via inhibition of TGF-β1 secretion as well as via inhibition of T cells' response to TGF-β1. This may provide an opportunity for developing a novel adjuvant therapeutic strategy for malignant gliomas, combining Scutellaria with immunotherapy and chemo/radio-therapeutic regimen, which could potentially improve the disease outcome.

Keywords Scutellaria · Flavonoids · Wogonin · TGF-β · Regulatory T cells · Glioma

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S. Dandawate · L. Williams · S. Mittal · P. Parajuli (✉)
Department of Neurosurgery, Wayne State University,
550 E. Canfield, Lande Bldg. #460, Detroit, MI 48201, USA
e-mail: pparajuli@med.wayne.edu

S. Dandawate
e-mail: dandawatesagar09@gmail.com

L. Williams
e-mail: linfwill@gmail.com

S. Mittal
e-mail: smittal@med.wayne.edu

N. Joshee
Agricultural Research Station, Fort Valley State University,
Fort Valley, GA, USA
e-mail: josheen@fvsu.edu

A. M. Rimando
USDA-ARS, Natural Products Utilization Research Unit,
University, MS, USA
e-mail: Agnes.Rimando@ARS.USDA.GOV

A. Thakur · L. G. Lum
Department of Oncology, Medicine and Immunology
and Microbiology, Karmanos Cancer Institute,
Wayne State University, Detroit, MI, USA
e-mail: thakur@karmanos.org

L. G. Lum
e-mail: luml@karmanos.org

Abbreviations

CM	Conditioned medium
ERK	Extracellular-regulated kinase
GSK	Glycogen synthase kinase
nT	Naïve T cells
IHC	Immunohistochemistry
SocL	*Scutellaria ocmulgee* leaf extract
T_{reg}	Regulatory T cells
TGF	Transforming growth factor

Introduction

Type of cytokines along with type and density of immune cells in the tumor microenvironment plays a critical role in determining the progression of malignant tumors [1–5]. High-grade gliomas are known to suppress immune responses via secretion of TGF-β1 [1, 6, 7]. All stages of anti-tumor immune response, including antigen presentation by dendritic cells, T-cell proliferation, and execution of cytolytic activity by cytotoxic T lymphocytes are inhibited by TGF-β [8–12]. Apart from direct suppression of immune cells, major immunomodulatory mechanism of TGF-β involves induction of FOXP3 in naïve T (nT) cells, leading to the generation of T_{reg} (inducible, iT_{reg}) cells [13–15]. TGF-β1 is also critical in the expansion of pre-existing T_{reg} (natural, nT_{reg}) cells [16, 17]. T_{reg} cells inhibit $CD4^+$ and $CD8^+$ T cells, DCs, NK cells and B cells via cell-to-cell contact and/or via secretion of cytokines such as TGF-β1 and IL-10 [6, 18]. Induction of T_{reg} cell activity and recruitment of T_{reg} cells into the tumor is one of the most critical mechanisms of immune-escape by malignant gliomas [1, 19–21]. It has also become evident that tumor-induced T_{reg} cell activity is responsible for the sub-optimal response to most therapeutic vaccines [1, 6, 22]. Development of neo-adjuvant strategies targeting TGF-β and T_{reg} cell activity is therefore imperative.

Extracts or flavonoids derived from traditional herb *Scutellaria* sp. have shown promise against malignant glioma in pre-clinical studies [23–27]. Monotherapy with an extract of Scutellaria has shown limited but encouraging results in patients with breast cancer [28, 29]. *To the best of our knowledge, there has been no study on the modulation of anti-tumor immune response by natural flavonoids*. However, a few encouraging studies have been reported on the tea polyphenol Epigallocatechin gallate (EGCG) and the soy isoflavone Genistein. Higher numbers of $CD8^+$T cells were detected in the EGCG-treated skin tumors, compared with non-EGCG-treated tumors in mice [30]. Chemotherapy with EGCG also enhanced $CD8^+$ T cell-mediated antitumor immunity induced by vaccination with vaccinia virus [31] or a DNA vaccine [32]. Genistein enhanced host resistance in the B16F10 tumor model, which may be related to increased activities of CTLs and NK cells [33]. *However, the mechanism of immune modulation by EGCG or genistein has not been reported*. Recent studies have reported the inhibition of TGF-β1 by selecting plant flavonoids in animal models of lung and liver fibrosis [34–36], implying that flavonoids may also inhibit TGF-β1-mediated T_{reg} cell activity in the tumor.

We have recently demonstrated in vivo efficacy of SocL extract in rat models of glioma [26]. In an earlier study, we reported that wogonin was the dominant flavonoid in SocL extract [27]. We report here that administration of *Scutellaria ocmulgee* leaf extract (SocL) reduced the number of infiltrating $FOXP3^+$ T_{reg} cells in gliomas, which correlated with an inhibition of TGF-β1 secretion by gliomas in vivo as well as in vitro. Moreover, SocL and a constitutive flavonoid wogonin also inhibited in vitro induction of T_{reg} activity in the presence of glioma-conditioned medium (CM) or TGF-β1. The results could have potential implication in developing adjuvant therapeutic strategy for malignant gliomas.

Materials and methods

Cell lines and flavonoid wogonin

Rat malignant glioma cell line F98 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM (low glucose) supplemented with 5% FBS. Wogonin, purchased from Wako Chemicals (Richmond, VA), was reconstituted in DMSO at 20 mM concentration and diluted in respective media before use.

Cultivation of Scutellaria plants and preparation of leaf (SocL) extracts

Scutellaria ocmulgee plants were cultivated at the 'Specialty Plants House' as described earlier [26, 27]. The leaves were harvested and shade-dried at room temperature until they lost about 80% moisture. The dried leaves were then ground, and extraction was performed using an ASE apparatus (Dionex Corporation, Sunnyvale, CA) as described earlier [26].

In vivo studies

The in vivo experiments have been described in details, and the results on tumor growth have been presented in our earlier study [26]. Briefly, a group of six F344 rats were transplanted with 1×10^6 F98 cells subcutaneously, in the right flank. After 5 days, when the tumor was palpable, the animals were divided into two groups. One group of

animals received SocL extract (100 mg/kg) via oral gavage in 500 µl of saline, once a day, 5 days a week, for 2 weeks. The control group received only saline. On day 29, after tumor transplantation, the animals were euthanized and the subcutaneous tumors were excised and then fixed in 4% PFA–PBS for immunohistochemistry.

In vitro T_{reg} cell induction model

In order to determine the effect of glioma-derived factors on the induction of T_{reg} cells, an in vitro model was developed by modifying, combining and appropriating two protocols [37, 38]. Briefly, PBL or MACS-isolated $CD4^+$ naïve T (nT) cells were cultured (1×10^6 cells/well) in a 6-well plate with anti-CD2/CD3/CD28-loaded MACS microbeads (Miltenyi Biotech, Auburn, CA) and low-dose IL-2 (50 IU/ml) in serum-free X-vivo medium (Lonza, Walkersville, MD). Tumor-mediated effects on immune activity were simulated by adding U87-MG glioma-conditioned medium (U87 CM) (50% v/v) or TGF-β1 (20 ng/ml) in the in vitro model. Based on our earlier studies [26, 27], all in vitro investigations were carried out with SocL extract and wogonin at 250 µg/ml and 60 µM concentrations, respectively, unless otherwise specified.

Functional analysis of T_{reg} cell activity: co-culture suppression assay

Freshly isolated $CD4^+CD25^-$ nT cells were used as responders. nT cells were resuspended in PBS (1×10^6 cells/ml) and stained with 0.2-µM CFSE for 1 h at 37°C. CFSE-labeled responder cells were plated (1×10^5 cells/well) in a 96-well round bottom plate, in the presence of anti-CD3- and anti-CD28-coated microbeads, along with IL-2 (50 IU/ml). iT_{reg} cells from various experimental groups were plated at 1:5 ratio with respect to the responder cells. The nT cells cultured for 7 days in the absence of TGF-β1 were used as *negative control* for iT_{reg} cells. The co-cultures were incubated for 5 days at 37°C and then analyzed by flow cytometry. Suppression of proliferation in the responder cells was evaluated using the proliferation module of the ModFit LT (Macintosh) software.

Cell proliferation assay

Cells were seeded in 96-well flat-bottom plates (2×10^4 cells/well) and cultured in the presence of SocL or wogonin as described elsewhere [26], with some modifications. Cell proliferation was assayed by using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI), which determines the number of viable cells in a culture by quantification of ATP. At the end of culture period, 100 µl of the CellTiter-Glo® reagent mix was added to 100 µl of culture volume and then the luminescence was measured with integration time set for 0.25–1 s using an Omega imaging system (UltraLum Inc., Claremont, CA). The cell proliferation was expressed as a percentage value of control cells cultured with medium alone.

Cytokine analysis

Cytokines were measured in the culture supernatants using a 25-plex human cytokine Luminex Array (Invitrogen, Carlsbad, CA) and Bio-Plex system (Bio-Rad Lab., Hercules, CA). The multiplex panel includes interleukin (IL)-2 and IL-10. The limit of detection for these assays is <10 pg/mL based on detectable signal of >2-fold above background (Bio-Rad). Cytokine concentration was automatically calculated from a standard curve by the BioPlex Manager Software (Bio-Rad). TGF-β1 was detected using an ELISA kit (BD Biosciences, San Jose, CA); in order to include the latent TGF-β in the measurement, the culture supernatants were acid-treated as per the manufacturer's protocol.

Immunohistochemistry (IHC)

A published protocol [26] was followed with some modifications. Briefly, the frozen or paraffinized tumor specimens were cut into 5-µm tissue sections, fixed in acetone for 5 min and stored at −80°C until staining time. For staining, slides were brought to room temperature, blocked and hydrated in staining buffer (PBS with 5% goat serum), and appropriate primary antibodies (anti-CD3, anti-CD4, anti-FOXP3 from eBioscience, San Diego, CA; anti-TGF-β1 from Santa Cruz Biotechnology, Santa Cruz, CA) were applied, followed by washing and incubation with the appropriate biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstaining with Mayer hematoxylin followed by dehydration and mounting. For co-localization, appropriate secondary antibodies conjugated with FITC or PE (Santa Cruz Biotechnology, Santa Cruz, CA) were utilized. The nucleus was stained with DAPI. Negative staining was performed with appropriate isotype control antibodies (eBioscience, San Diego, CA) instead of the specific primary antibody. Lymph node tissues obtained from a normal rat were used as a positive control for T_{reg} staining. The sections were then analyzed under a fluorescent microscope (Olympus BX51) equipped with a digital camera and micrographed at 200× or 400× magnification, as indicated.

Western blot analysis

Western blot analysis of the protein samples were performed as described elsewhere [26]. Briefly, 20–30 µg

aliquots of total protein were electrophoresed, transferred onto PVDF membrane and probed with specific antibodies against phosphorylated and non-phosphorylated forms of signaling/transcription molecules (Cell signaling Technology, Danvers, MA). Detection of HRP-conjugated Abs was performed using SuperSignal (Pierce, Rockford, IL), and chemiluminescence was recorded using an Omega imaging system (UltraLum Inc., Claremont, CA).

Statistical analysis

The ex vivo analysis of tumor and peripheral blood was repeated with samples from three animals per group. The in vitro experiments were also replicated at least three times. A Wilcoxon's log-rank test was performed to determine the statistical difference between various experimental and control groups, using the SPSS package (SPSS Inc, Chicago, IL). A '*P*' value less than 0.05 was considered significant.

Results

SocL preferentially inhibits intra-tumoral FOXP3$^+$ T_{reg} cell activity without affecting total T-cell infiltration

We have recently reported in vivo efficacy of SocL extract using F38 glioma cells transplanted in F344 rats. F344 rats were subcutaneously transplanted with 1×10^6 F98 glioma cells. Animals were administered with SocL extract as described [26]. After 29 days of tumor transplantation, animals were euthanized; the subcutaneous tumor was resected and processed for IHC. As shown in Fig. 1, tumors obtained from animals treated with SocL extract had significantly reduced the infiltration of CD4$^+$FOXP3$^+$ T_{reg} cells (Fig. 1b) compared to the control tumor (Fig. 1a). On the other hand, there was no significant difference in anti-CD3 staining in SocL-treated tumors (Supplementary Figure S1D) compared to the controls (Figure S1C). These data indicate that SocL selectively inhibits the infiltration or expansion of T_{reg} cells in the tumor without significantly affecting other T-cell subsets.

SocL and wogonin inhibit the secretion of TGF-β1 by malignant gliomas in vitro as well as in vivo

Tumor-derived TGF-β has been implicated in the induction/expansion of T_{reg} cells. Therefore, we investigated whether SocL-mediated reduction in tumor-infiltrating T_{reg} cells was associated with a concomitant reduction in TGF-β1 secretion by the tumors. IHC analysis of F98 glioma, obtained after 29 days of subcutaneous transplantation in F344 rats as described above, showed profuse TGF-β1 staining (Fig. 2a), which was significantly reduced in tumors obtained from animals treated with SocL (Fig. 2b). Moreover, peripheral blood from untreated F98-bearing rats, collected after 29 days of tumor transplantation, contained more than 2 ng/ml of TGF-β1, which was significantly reduced to 0.6 ng/ml in rats treated with SocL (Fig. 2c).

We also examined the inhibition of TGF-β1 secretion by human (U87-MG) and rat (F98) gliomas following treatment with SocL and wogonin in vitro. SocL and wogonin, at 250 μg/ml and 60 μM respectively, significantly inhibited the secretion of TGF-β1 in both U87-MG and F98 glioma cells, as determined by ELISA after 72 h of culture (Fig. 2d). These data suggest a role for the inhibition of TGF-β by Scutellaria in delaying the growth of glioma in vitro as well as in vivo.

SocL and wogonin inhibit TGF-β1-mediated induction of T_{reg} activity in vitro

These set of experiments examined the role of glioma-derived factors in the induction of T_{reg} phenotype and activity, and inhibition thereof by SocL or wogonin. CD4$^+$ nT cells were cultured in the T_{reg} induction model, in the presence or absence of TGF-β1 or U87-MG CM (50% v/v) as described in the Materials and Methods section. After 7 days of culture, the cells were analyzed by flow cytometry. All cells were gated on CD4-FITC. The medium control group contained less than 3.4% CD4$^+$CD25$^+$ FOXP3$^+$ (T_{reg}) cells (Fig. 3a). Addition of TGF-β1 as well as U87-MG CM significantly increased the frequency of T_{reg} cells (19%, Fig. 3b and 17% Fig. 3e, respectively). Both SocL and wogonin significantly inhibited the frequency of T_{reg} cells induced by TGF-β1 (10 and 1.9%, Fig. 3c, d, respectively) as well as those induced by U87-MG CM (8.6 and 5.1%, Fig. 3f, g, respectively). Moreover, U87-MG CM-mediated induction of T_{reg} cell frequency was significantly inhibited upon addition of anti-TGF-β1 (3.8%, Fig. 3h vs. 17%, 3e), indicating that TGF-β1 secreted by the glioma cells was responsible for induction of T_{reg} phenotype in the nT cell cultures. Together with data presented in Fig. 1, these results strongly indicate that SocL and wogonin inhibit glioma-mediated induction of T_{reg} phenotype via inhibition of TGF-β1 activity.

We next examined whether in vitro inhibition of T_{reg} cell phenotype by SocL and wogonin also corresponds to an inhibition of T_{reg} cell function via co-culture suppression assay, as described in the Methods section. The 'control' nT cells, cultured with anti-CD2/3/28-labeled beads and 50 U/ml IL-2, showed high rate of proliferation (Fig. 4a, proliferation index 8.8 with 0.7% undivided cells and 59% cells in generations 5 and beyond). TGF-β1-induced T_{reg} cells significantly inhibited the proliferation of

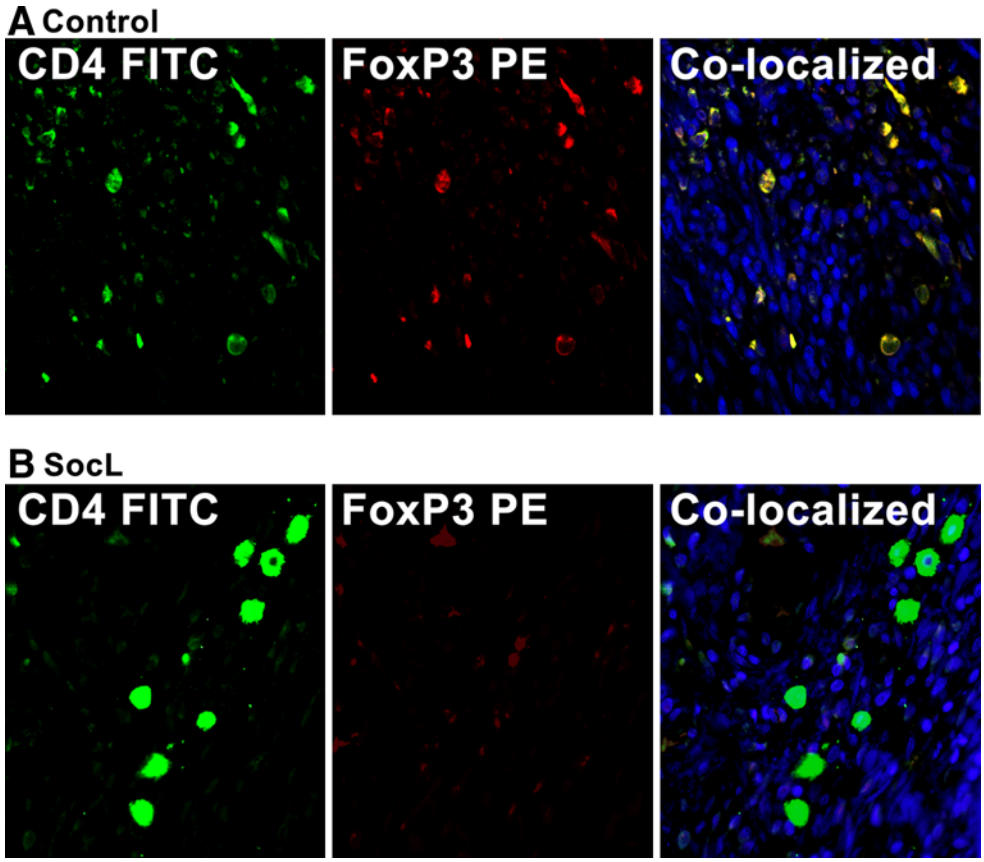

Fig. 1 SocL preferentially inhibits intra-tumoral FOXP3$^+$ T_{reg} cell activity without affecting total T-cell infiltration. F344 rats were subcutaneously transplanted with 1×10^6 F98 glioma cells on the *right* flank. SocL administration was performed as described. Rats were euthanized on day 29; the tumor was resected and processed for fluorescent IHC, as described in the "Materials and methods" section, to determine the presence of CD4$^+$FOXP3$^+$ T_{reg} cells. Result shown is from one representative paraffinized glioma specimen out of three studied. The micrographs were imaged at 400× magnifications

nT cells (Fig. 4b, proliferation index 5.8 with 4.5% undivided cells and only 24% cells in generations 5 and beyond). On the other hand, nT cells cultured with T_{reg} cells induced in the presence of SocL or wogonin showed proliferation comparable to the control groups (Fig. 4c, d vs. Fig. 4a, respectively), demonstrating an inhibition of T_{reg} 'activity' by SocL and wogonin.

Treatment with SocL and wogonin resulted in an observed increase in IL-2 and a concomitant decrease in IL-10 levels in the T_{reg} cell culture

In the next experiment, nT cells were cultured in T_{reg} cell induction model under the same conditions as described in the previous experiment; the culture supernatant was analyzed for IL-2 and IL-10 secretion, at various time-points, using the cytokine multiplex assay. We observed a considerable but statistically non-significant increase in the frequency of IL-10 (Fig. 5a) with a similar, concomitant decrease in IL-2 (Fig. 5b) following 72 h of T-cell culture with TGF-β1, compared to the medium control groups. Treatment with SocL and wogonin significantly reversed TGF-β1-mediated enhancement or inhibition of IL-10 and IL-2 (Fig. 5a, b), respectively. Statistically significant differences in cytokine levels between TGF-β1 and TGF-β1 + SocL/wogonin groups were observed after 72 h of culture. These results, together with results shown in Figs. 2 through 4, clearly demonstrate that SocL and wogonin inhibit TGF-β1-induced phenotypic or functional (cytokine) responses in T cells.

SocL and wogonin inhibit the phosphorylation of Smad3, GSK-3β and p44/42 while enhancing the phosphorylation of p38 MAPK in T cells in vitro

We tested whether SocL extract and wogonin-mediated inhibition of T_{reg} activity involved modulation of TGF-β-related Smad or non-Smad signaling. T_{reg} cell culture was performed as described in the "Materials and methods" section. After 7 days, the cells were lysed, electrophoresed and analyzed by western blot. As shown in Fig. 4, TGF-β1 enhanced the phosphorylation of Smad3 in the T_{reg} cells,

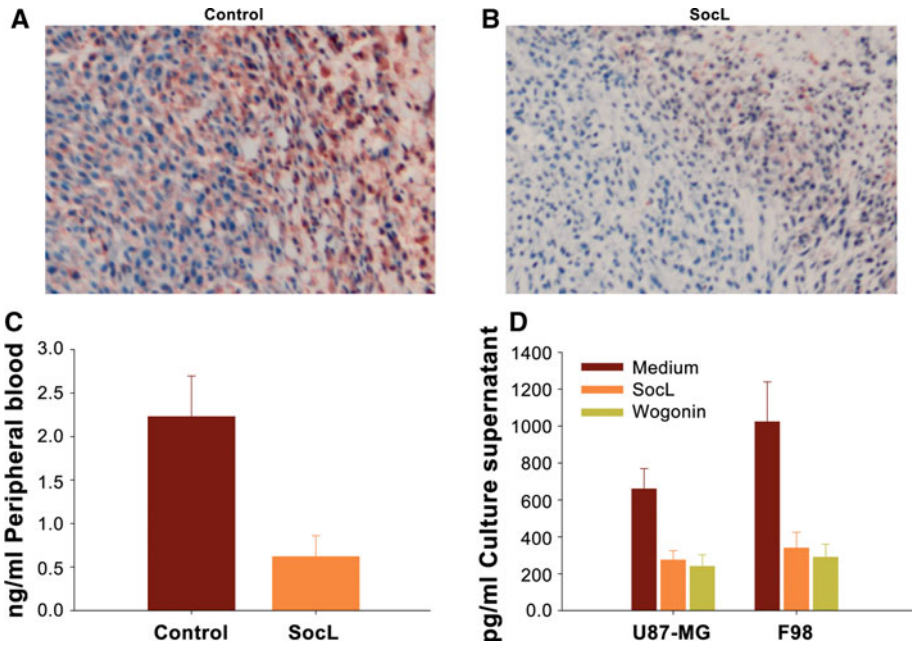

Fig. 2 SocL and wogonin inhibit the secretion of TGF-β1 by malignant gliomas in vitro as well as in vivo. (**a**, **b**) F344 rats were subcutaneously transplanted with 1×10^6 F98 glioma cells on the *right* flank. SocL administration was performed as described. Rats were euthanized on day 29; the tumor was resected and processed for IHC, as described in the "Materials and methods" section, to detect TGF-β1. Result shown is from one representative paraffinized glioma specimen out of three studied. The micrographs were imaged at 200× magnifications. **c** Peripheral blood obtained from F344 rats 29 days after F98 glioma transplantation, with or without SocL administration, was analyzed for the secretion of TGF-β1 by ELISA. The data are expressed in ng/ml of CM. **d** Conditioned media (CM) from 72-h culture of a human (U87-MG) and a rat (F98) glioma cell line were analyzed for the secretion of TGF-β1 by ELISA. The data are expressed in pg/ml of CM. Result shown is from one representative experiment out of three performed with similar results

which was significantly inhibited following treatment with SocL extract and wogonin. Similarly, SocL extract and wogonin also inhibited the phosphorylation of p44/42 MAPK, driven by the presence of TGF-β1. Interestingly, phosphorylation of GSK-3β was already evident in the control cultures, which as marginally inhibited by TGF-β1. Treatment with SocL and wogonin in the presence of TGF-β1 further inhibited the phosphorylation of GSK-3β. On the other hand, control T cells also showed considerable phosphorylation of p38 MAPK, which was, however, significantly inhibited by TGF-β1; both SocL and wogonin significantly enhanced the phosphorylation of p38 MAPK compared to cells treated with TGF-β1 alone (Fig. 6). These results suggest that SocL and wogonin subdue the T cells' response to TGF-β1 via inhibition of TGF-β1-induced phosphorylation of Smad3 and probably also via modulation of non-Smad signaling.

Discussion

Immunotherapeutic strategies in high-grade gliomas have met with limited success in the clinic because of glioma-mediated active immune suppression [6, 22, 39]. TGF-β1, which is arguably the most potent immunosuppressive cytokine, has been reported in the tumor and in the serum of most glioma patients. Apart from directly inhibiting the functions of DC, NK and CTLs, TGF-β1 plays a critical role in the induction and expansion of T_{reg} cells in the tumor as well as in the periphery. $FOXP3^+$ T_{reg} cells are major contributors in limiting the efficacy of therapeutic cancer vaccines [6, 22, 40]. Immunotherapy along with complete depletion of T_{reg} cells, however, often leads to undesirable autoimmune phenomena [41, 42]. Therefore, development of novel adjuvant strategies to counter tumor-mediated immune suppression is not only desirable but necessary for combination therapy to be successful in the treatment of malignant gliomas.

The principal flavonoids found in Scutellaria extracts are baicalein, its derivative baicalin and wogonin; while the presence of apigenin, chrysin, scutellarin and luteolin has also been reported [27, 43]. In an earlier study, we reported that wogonin was the dominant flavonoid in SocL extract [43]. *There have been few studies using Scutellaria or constituent flavonoids for the treatment of gliomas*. A recent study has illustrated anti-proliferative effects of an ethanolic extract from *S. baicalensis* root extract in drug-resistant gliomas when used alone and in conjunction with bis-chloroethylnitrosourea (BCNU) [23]. Flavonoid baicalein has also shown a favorable effect in cisplatin-induced

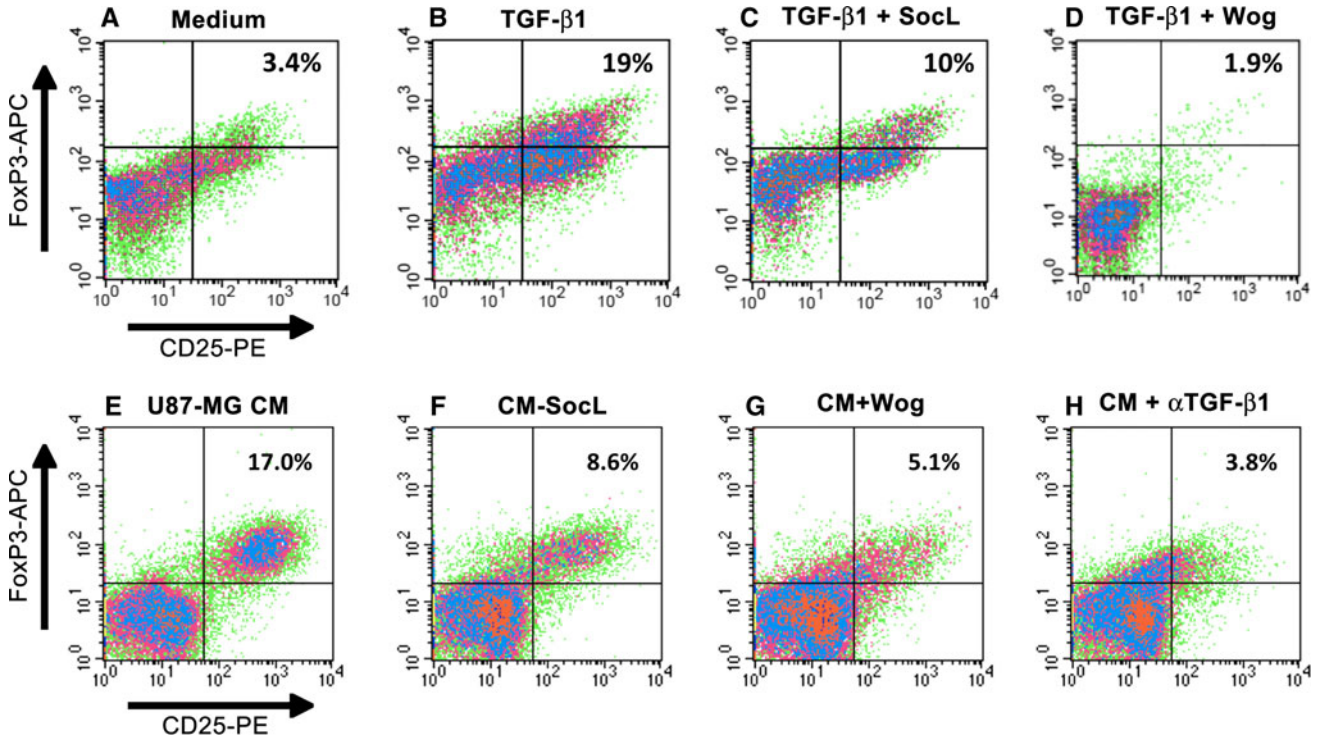

Fig. 3 Glioma CM induces T_{reg} cells, which is inhibited by SocL, wogonin and anti-TGF-β1. nT cells, isolated from the PBL of normal donors, were cultured in the T_{reg} induction model with recombinant TGF-β1 or U87-MG CM, in the presence of SocL, wogonin or anti-TGF-β1, as indicated. After 7 days of culture, the cells were analyzed by flow cytometry as described in the "Materials and methods" section. The data are from one representative experiment out of three experiments performed with similar results

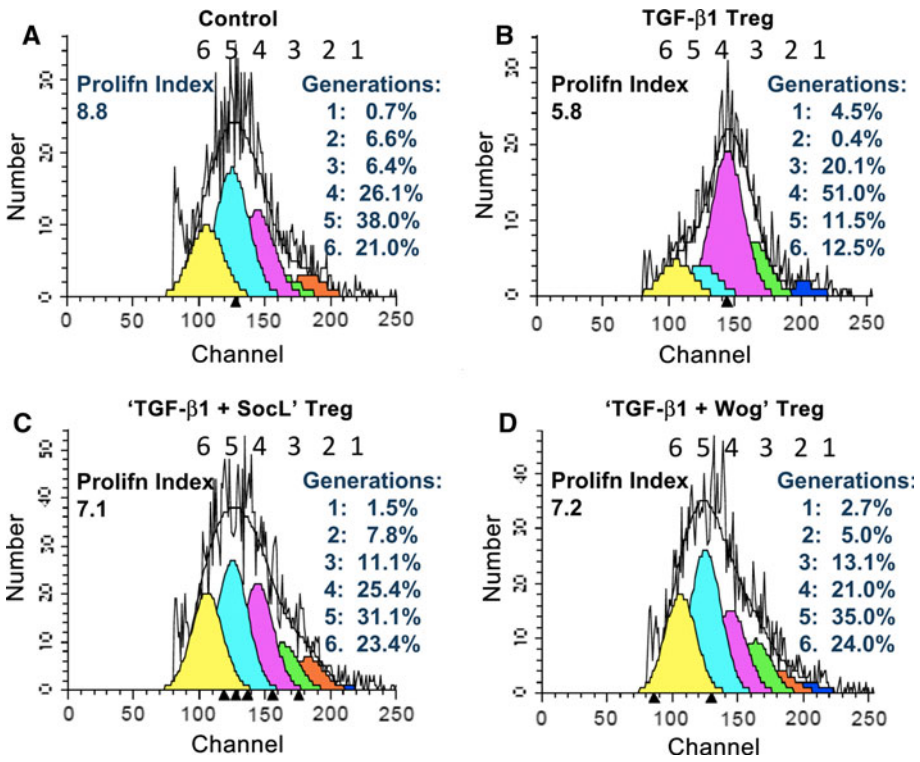

Fig. 4 SocL extract and wogonin inhibit TGF-β1-mediated induction of T_{reg} activity in vitro. The nT cells, isolated as described above, were either cultured with anti-CD2/3/28-labeled beads and 50U/ml IL-2 alone (**a**, control) or with T_{reg} cells obtained from cultures containing TGF-β1 **b**, TGF-β1 + SocL **c** or TGF-β1+ wogonin **d**, at nT/Treg ratio of 5:1. After 5 days, the cells were analyzed by flow cytometry. Results are expressed in proliferative index as well as the frequency of cells in various cell cycle generations, as determined by the proliferation module of the ModFit LT software. The data are from one representative experiment out of two performed with similar results

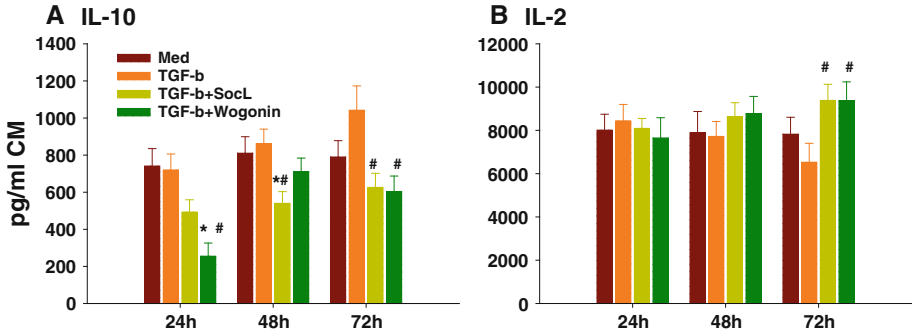

Fig. 5 Treatment with SocL and wogonin results in an observed increase in IL-2 and a concomitant decrease in IL-10 levels in the T_{reg} cell culture. The nT cells were cultured in the T_{reg} induction model with TGF-β1, in the presence of SocL or wogonin, as indicated. After 24, 48 and 72 h, the culture supernatants were harvested and the cytokine levels were determined using a Bioplex assay as described in the "Materials and methods" section. The data, expressed in pg/ml of culture supernatant, are from one representative experiment out of two performed with similar results. $^{*}P < 0.05$ versus medium control; $^{\#}P < 0.05$ versus cultures treated with TGF-β1 alone

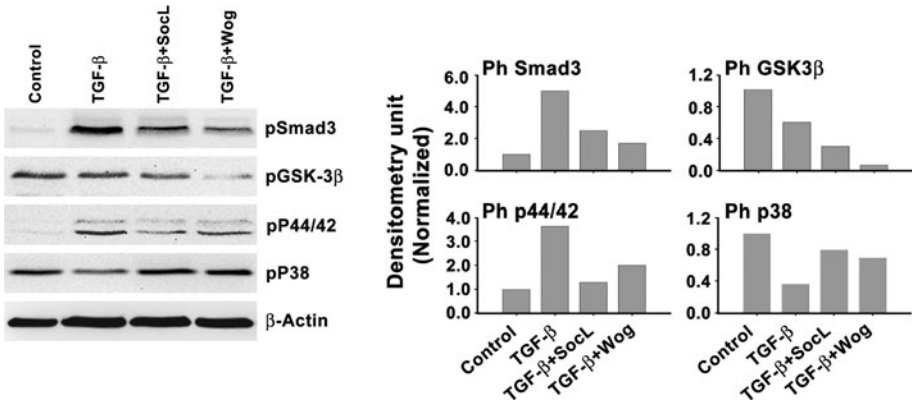

Fig. 6 SocL and wogonin inhibit the phosphorylation of Smad3, GSK-3β and p44/42 while enhancing the phosphorylation of p38 MAPK in T cells in vitro. The nT cells were cultured in the T_{reg} induction model with TGF-β1, in the presence of SocL or wogonin, as indicated. After 7 days, the cells were lysed, electrophoresed and subjected to western blotting. Grayscale density was analyzed using ImageJ software, and values for phosphorylated signaling molecules were normalized against those for β-Actin. Data are representative of three experiments performed with similar results

cell death of human glioma cells [25]. Our group has recently reported in vivo efficacy of SocL extract in rat models of glioma [28]. Two phase I clinical trials have recently been concluded using BZL101, an aqueous extract from *Scutellaria barbata*, in patients with advanced breast cancer [28, 29]. These studies showed promising clinical activity for the Scutellaria extract in heavily pre-treated patients with metastatic breast cancer. Immunologic status of these patients was not evaluated.

Scientific studies on immunomodulation by extracts or isolated active components from Scutellaria, or for any herbal flavonoid, have been mostly limited to their anti-inflammatory activities [44–46]. These reports often lead to the notion that flavonoids are immune-suppressive. However, apart from occasional, mild and transient lymphopenia, no major hematologic toxicity or immune suppression has been reported in pre-clinical or clinical studies using flavonoids [47]. On the other hand, flavonoid-mediated recovery from hypoxia-induced lymphopenia has been reported [48]. Interestingly, wogonin has been shown to remove immune suppression without affecting inflammatory response to glucocorticoids [49]. Wogonin has also been shown to enhance tumor necrosis factor activity in RAW 264.7 macrophage cells [50]. We observed reduced intra-tumoral T_{reg} cell activity in SocL extract–treated animals, while total $CD3^{+}$ or $CD4^{+}$ T cell frequency was unchanged.

Our studies showed reduced frequency of intra-tumoral T_{reg} cells following treatment with SocL, which was associated with a concomitant decrease in TGF-β1 in the tumor. We also observed significant inhibition of TGF-β1 secretion by both human and rat gliomas, following treatment with SocL and wogonin, in vitro as well as in vivo. TGF-β1 has been known to induce T_{reg} phenotype in naïve T cells and also induces the expansion of pre-existing T_{reg} cells [13–17]. Our results showed considerable secretion of TGF-β1 by human glioma (U87-MG) cells and rat glioma (F98) in vitro as well as in vivo. These data clearly

indicated that Scutellaria flavonoids could inhibit glioma-induced T_{reg} activity via inhibition of TGF-β1 secretion, at least in part.

The second part of our studies sought to determine whether SocL or wogonin could also inhibit T_{reg} activity by a direct inhibition of the T cells' response to TGF-β1. We observed significant induction of T_{reg} activity by recombinant TGF-β1, which was inhibited by both SocL extract and wogonin. A comparable induction of T_{reg} activity was also observed in the presence of U87-MG CM, which was also inhibited by Scutellaria flavonoids as well as by anti-TGF-β1 antibody. Western blot analysis further revealed clear modulation of TGF-β1-induced signaling in the T_{reg} cells in the presence of SocL and wogonin.

The canonical TGF-β1 signaling involves activation of the SMAD complex [51]. In addition to activation of SMAD, TGF-β1 signaling also involves activation of extracellular-regulated kinases (ERK 1/2) and p38 MAP kinases [52, 53]. The role of non-SMAD signaling in the induction of $FOXP3^+$ T_{reg} cells is controversial. Some earlier studies have indicated a requirement for both ERK 1/2 and p38 activation in TGF-β1-mediated iT_{reg} induction. On the other hand, a recent study by Lu et al. has shown that induction of T_{reg} phenotype in naïve T cells by TGF-β1 involves ERK 1/2, while p38 was not necessary in the process [54]. Our studies are in agreement with that of Lu et al. and revealed that inhibition of TGF-β1-induced T_{reg} activity by SocL or wogonin may involve, to some extent, an inhibition of ERK 1/2 (P44/42) MAPK activity.

We have earlier reported an inhibition of Akt and GSK-3β phosphorylation by Scutellaria flavonoids in malignant gliomas [26]. Recent reports in non-tumor models have shown that Akt and GSK-3β can directly interact with Smad3 and regulate its transcriptional activity [55–57]. We observed significant inhibition of TGF-β1-induced Smad3 phosphorylation by SocL or wogonin in T_{reg} cell cultures, which was associated with a concomitant inhibition of GSK-3β phosphorylation. Therefore, it is likely that inhibition of TGF-β1 activity by SocL or wogonin in T cell cultures is mediated by their regulation of AKT/GSK3β signaling. Whether SocL or wogonin could directly inhibit Smad3 phosphorylation, remains to be studied.

It is becoming increasingly clear that the initial anti-tumor effects of Scutellaria (via TGF-β1 blockade or otherwise) can be overcome by tumor burden, as observed in our own experiments with intracranial tumor models [26] as well as in other pre-clinical and clinical studies [23, 28, 29]. These studies have indicated a clear need for combining Scutellaria with other chemo- or immunotherapeutic approaches. Our current study demonstrated that Scutellaria extract could preferentially inhibit intra-tumoral T_{reg} activity without significantly affecting the frequency of total T cells. Moreover, our in vitro studies with co-culture inhibition indicated that SocL or wogonin may, to some extent, relieve T_{reg}-mediated immune suppression. The cytokine analysis further revealed an inhibition of immune-suppressive IL-10 secretion by SocL and wogonin, whereas the level of IL-2 was either unchanged or marginally enhanced. Overall, the present study suggests that SocL extract or wogonin can potentially reverse tumor-mediated immune suppression via inhibition of TGF-β1 secretion as well as via inhibition of the T cells' response to TGF-β1. This may provide an opportunity for developing a novel adjuvant therapeutic strategy for malignant gliomas, combining Scutellaria or constituent flavonoids with immunotherapy and chemo/radio-therapeutic regimen, which could potentially improve the disease outcome.

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

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