

ORIGINAL RESEARCH

Sophoraflavanone G decreases the migratory ability of cancer cells by altering the cytokine profile of the environment in endometrial cancer and macrophage co-culture

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Abstract

Background: Macrophages play key roles in initiating, sustaining, and resolving inflammation. *Sophora flavescens* has traditionally been used as a herbal anti-inflammatory treatment for inflammation-related conditions. This study aimed to investigate how sophoraflavanone G (SF-G) modulates the inflammatory response in RAW264.7 macrophages when co-cultured with Ishikawa endometrial cancer cells. **Methods:** The cytotoxicity of SF-G in RAW264.7 cells was assessed using the Cell Counting Kit-8 (CCK-8) assay. Gene expression analyses of inflammatory markers in macrophages and migration-related genes in co-cultured endometrial cancer cells were performed using quantitative Real-time Polymerase Chain Reaction (qRT-PCR). Protein levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA). Unstimulated RAW264.7 cells were used as the control group. **Results:** Co-culture with cancer cells significantly increased the expression of pro-inflammatory genes (Interleukin 1 beta (*IL1β*), Interleukin 6 (*IL6*), Cluster of Differentiation 86 (*CD86*), and Transforming Growth Factor beta (*TGFβ*)) in macrophages. Treatment of macrophages with SF-G significantly upregulated anti-inflammatory markers (Cluster of Differentiation 163 (*CD163*) and Interleukin 10 (*IL10*)) during co-culture with cancer cells. Furthermore, the migratory ability of Ishikawa cells was markedly reduced in the co-culture with SF-G-treated macrophages compared with the co-culture without SF-G. **Conclusions:** Overall, SF-G treatment altered the inflammatory response of RAW264.7 macrophages in the co-culture microenvironment and inhibited the migration of Ishikawa endometrial cancer cells.

Keywords

Macrophage; Endometrial cancer; Inflammation; Migration

1. Introduction

In East Asian countries, the roots of *Sophora flavescens* have long been used as a herbal anti-inflammatory treatment for atopic dermatitis, chronic bronchitis, acne, and other inflammation-related conditions [1]. *Sophora flavescens* contains over 50 prenylated flavonoids that have been isolated and identified to date [2], and it has been reported to contain a variety of flavonoids such as kurardin, kurarinone, kushenol B, formononetin, and sophoraflavanone G [3, 4]. Sophoraflavanone G (SF-G; 5,7-D, 2',4'-tetrahydroxy-8-lavandulylflavanone) inhibits tyrosinase and glycosidase activities and has different biological activities, such as anti-inflammatory and anti-cancer effects [5–7].

Endometrial cancer (EC) is a prevalent malignant neoplasm in gynecology. The prevalence and mortality rates of EC have been steadily rising worldwide, and in certain affluent nations, it is now the most common malignancy in the female reproduc-

tive system, particularly among postmenopausal women [8]. A significant percentage of patients present with advanced-stage disease due to the subtle onset of EC, which frequently has non-specific or absent early symptoms. This significantly impairs treatment efficacy and prognosis [9]. According to recent studies, a key factor in this is the modulation of the tumor niche. Tumor cells, immune cells, cytokines, and other components make up what is called the tumor microenvironment (TME), which promotes tumor growth by a variety of mechanisms; the anti-tumor immune response is weakened by immunosuppressive cells (Tregs, M2 macrophages) and extracellular matrix remodeling encourages invasion and metastasis [10–12].

Macrophages regulate numerous homeostatic and evolutionary host-defense immune responses, making them essential for innate immunity. The three primary roles of macrophages are antigen presentation, phagocytosis, and immunomodulation. They are necessary for the execution of typical immune re-

sponses under various pathological conditions [13, 14]. The fate of macrophages depends on several external factors that promote polarization toward either the pro-inflammatory M1-type or the alternatively activated M2-type immune response. Macrophage M1 or M2 polarization is a carefully controlled process involving multiple important signaling pathways, post-transcriptional regulatory networks, and epigenetic mechanisms [15–17].

In this study, SF-G was applied to macrophage cells co-cultured with endometrial cancer cells in order to examine the polarization status. Furthermore, the effect of SF-G-treated macrophages on the migration capacity of cancer cells in cancer-macrophage co-culture was investigated.

2. Materials and methods

2.1 Chemicals

Sophoraflavanone G (Cat#HY-N1231 MedChemExpress, Monmouth Junction, NJ, USA) was purchased commercially.

2.2 Cell culture

The RAW264.7 (ATCC® TIB-71™) murine macrophage cell line was obtained from the American Type Culture Collection (ATCC). The Ishikawa endometrial cancer cell line (Cat#

99040201) was obtained from the European Collection of Authenticated Cell Cultures (ECACC). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine at 37 °C in a humidified incubator with 5% CO₂. The cells were utilized in later experiments upon achieving 80% confluence.

2.3 Co-culturing of RAW264.7 and Ishikawa cells

To analyze gene expression and measure cytokine levels, monocultured cells were first prepared and then indirectly co-cultured. Fig. 1 illustrates the co-culture setup and SF-G treatment. A total of 5×10^4 Ishikawa cells were seeded into the 6-well plate. At the same time, 5×10^4 RAW264.7 cells were seeded into 0.4 μ m Corning® Transwell® inserts (Cat# CLS3450, Sigma-Aldrich, USA) in DMEM medium. Both cell types were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. RAW264.7 cells in the inserts were then treated with SF-G for 4 h. After the 4-hour treatment, the SF-G-containing medium was replaced with fresh medium without SF-G. The inserts were then transferred to the 6-well plates containing Ishikawa cells. After 24 h of co-culture, cells were collected from each well for mRNA isolation.

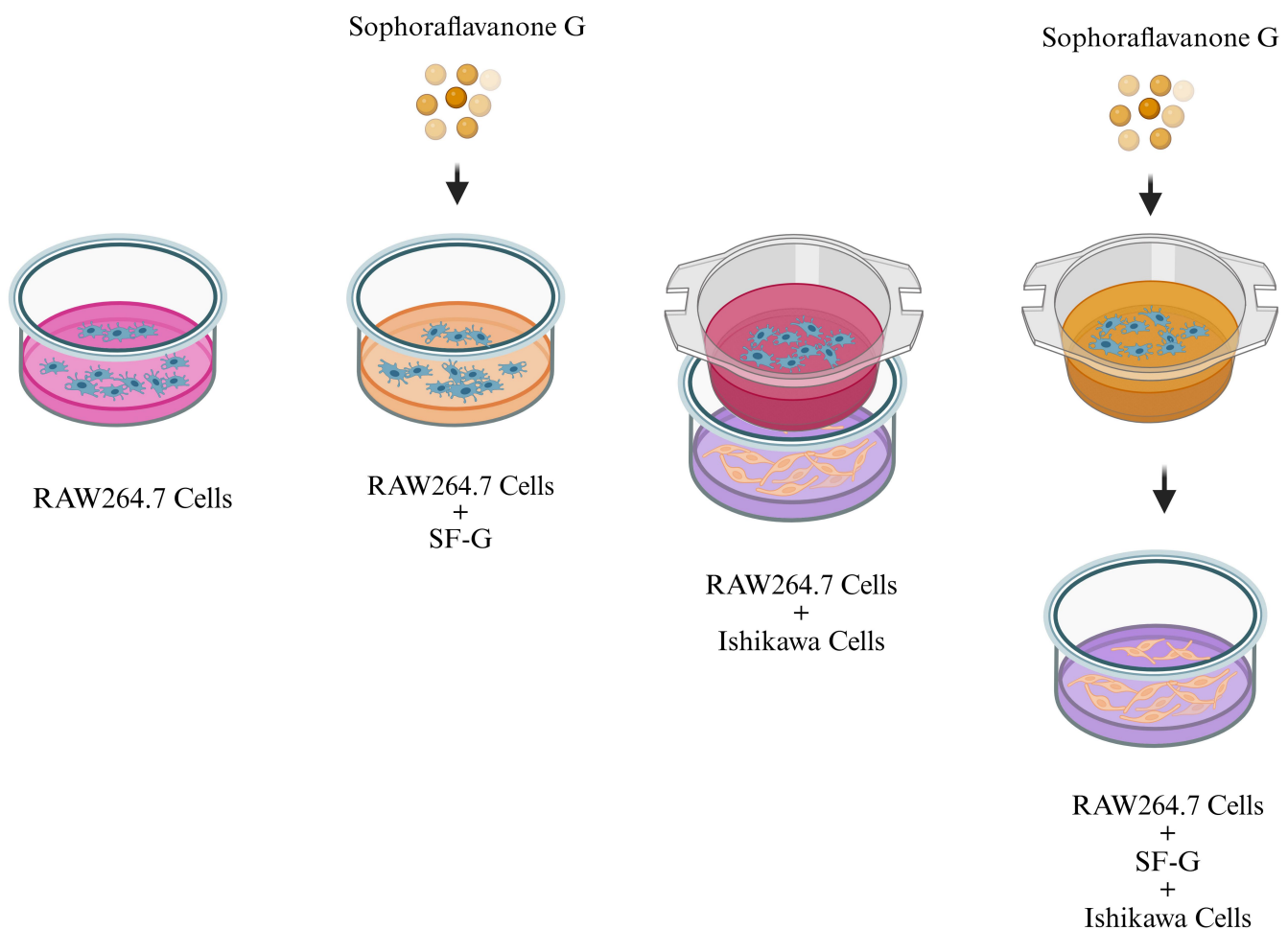


FIGURE 1. Schematic representation of co-cultures of RAW264.7 and Ishikawa cells used in the study. Created in <https://BioRender.com>. SF-G, sophoraflavanone G.

2.4 Cell counting assay

RAW264.7 cells (4000 cells per well) were seeded in a 96-well cell culture plate (Corning, Sigma-Aldrich, USA). A series of SF-G concentrations diluted in culture medium were then added to the plates and incubated for 24, 48, and 72 h at 37 °C. Three replicates of each measurement were made. After the 37 °C incubation period, 10 µL of the Cell Counting Kit-8 (CCK-8) reagent (Cat#HY-K0301, MedChemExpress, Monmouth Junction, NJ, USA) were added to each well. Two hours later, a multifunction microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland) was used to measure each well's optical density (OD) at 450 nm. For every concentration, cell viability was calculated as a percentage of the corresponding negative control mean.

2.5 RNA isolation and cDNA synthesis

According to the manufacturer's instructions, QIAzol (Qiagen, Cat#79306, Hilden, NRW, Germany) was used to isolate the total RNA from RAW264.7 and Ishikawa cells, and the Transcriptor First-Strand iScript™ cDNA Synthesis Kit (Cat#170-8851, Bio-Rad, Hercules, CA, USA) was used to synthesize cDNA.

2.6 Gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

Integrated DNA Technologies (IDT) PrimerQuest tool (online version, Coralville, IA, USA) was used to design the primer sequences for the inflammation- and cell migration-related genes analyzed by qRT-PCR in this study, and the results are shown in Table 1 [17, 18]. For each gene, a qRT-PCR reaction mix was prepared. In summary, each reaction contained 5 pmol of forward and reverse primers, 5 µL of Bio-Rad 2X SYBR Green Supermix, and 2 µL of cDNA. The qRT-PCR protocol

consisted of an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. qRT-PCR analysis was performed using the Bio-Rad CFX Connect™ Real-Time System. After amplification, a melting curve analysis was performed by gradually increasing the temperature from 65 °C to 95 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control in all qRT-PCR experiments.

2.7 Measurement of inflammatory mediators

The supernatants were collected from cultures of the cells. IL1β (Cat#SRB10141) and IL6 (Cat#SRB10134) levels were measured using SunRedBio ELISA kits. Following centrifugation of the supernatants for 20 minutes at 3000 rpm, 10 µL of the samples and 40 µL of dilution buffer were added to 96-well plates. Following 30 minutes of incubation at 37 °C, the wells were washed five times for 30 seconds each. 50 µL of horseradish peroxidase (HRP)-conjugated reagent were added to each well and then incubated for 30 minutes at 37 °C. After washing, added to each well 50 µL of chromogen solutions A and B, which were then gently shaken. After a 15-minute incubation at 37 °C in the dark, 50 µL of stop solution was added to terminate the reaction. The absorbance at 450 nm was then measured and recorded.

2.8 Measurement of nitric oxide (NO)

NO levels were measured using General Nitric Oxide Assay Kit (Cat#MBS8243214, MyBioSource, San Diego, CA, USA). RAW264.7 cells were collected from culture plates and the centrifuged supernatant was discarded. 500 µL of distilled water was added and homogenized. It was centrifuged at 12,000g for 20 minutes at 4 °C. 250 µL of assay buffer 1 and

TABLE 1. Primers used in this study.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	PCR (bp)
<i>IL1β</i>	GATCCCAAACAATACCCAAAGAAG	AGGTGCTGATGTACCAGTTG	118
<i>IL6</i>	CTTCCATCCAGTTGCCTTCT	CTCCGACTTGTGAAGTGGTATAG	134
<i>IL10</i>	CTATGCTGCCTGCTCTTACTG	GGGAAGTGGGTGCAGTTATT	83
<i>TGFβ</i>	CTGAACCAAGGAGACGGAATAC	GGGCTGATCCCGTTGATT	101
<i>CD86</i>	GGGCTTGGCAATCCTTATCT	CAGCTCACTCAGGCTTATGTT	139
<i>CD163</i>	CAGACTGGTTGGAGGAGAAATC	CAGCTTCCAGAGACAAGTCAA	101
<i>VIM</i>	TCCAAGCCTGACCTCAC	CACCTGTCTCCGGTACTC	189
<i>CDH1</i>	GAGAGCGGTGGTCAAAGAG	AGCTGGCTCAAGTCAAAGT	117
<i>CDH2</i>	GCTGACCAGCCTCCAAC	CATGTGCCCTCAAATGAAACC	112
<i>FN1</i>	CCCTAAAGGACTGGCATTCA	CTCGAGTAGGTCACCCTGTA	102
<i>TWIST1</i>	CCGCAGTCTTACGAGGAG	TTGCTCAGCTTGTCCGA	137
<i>GAPDH</i>	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	307

IL1β, interleukin 1 beta; *IL6*, interleukin 6; *IL10*, interleukin 10; *TGFβ*, transforming growth factor beta; *CD86*, cluster of differentiation 86; *CD163*, cluster of differentiation 163; *VIM*, vimentin; *CDH1*, cadherin 1 (E-cadherin); *CDH2*, cadherin 2 (N-cadherin); *FN1*, fibronectin 1; *TWIST1*, twist family bHLH transcription factor 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

250 μL of assay buffer 2 were added and centrifuged at 10,000g for 10 minutes at 4 °C. The supernatant was then taken into a new tube and 50 μL of sample was put into each well of a 96 well plate. 50 μL of Dye reagent A was added and incubated for 10 minutes. Then, 50 μL Dye reagent B was added and incubated for 5 minutes. The absorbance was measured at 550 nm OD and recorded.

2.9 Statistical analysis

The $2^{-\Delta\Delta C_t}$ method was used to determine the relative fold changes in gene expression levels for the qRT-PCR analysis. The relative expression levels of inflammation and migratory related genes were adjusted to GAPDH in qRT-PCR assays. The statistical analysis of gene expression results and ELISA data was conducted using the GraphPad Prism Software, version 8.0.2 (GraphPad Software, Boston, MA, USA).

3. Results

3.1 SF-G dose-dependently reduced the viability of macrophage cells

RAW264.7 cells were treated with different concentrations of SF-G (0–200 μM) for 24, 48, and 72 h. The viability of macrophage cells diminished with increasing dosage (Fig. 2). Using CompuSyn software, 20% inhibitory concentration (IC_{20}) dose of SF-G for RAW264.7 cells was determined to be 4.3 μM at 24 h. Consequently, RAW264.7 cells were subjected to treatment with SF-G at a concentration equivalent to the IC_{20} value in the subsequent tests.

3.2 Cytokine genes expression profile of macrophage cells was changed w/wo SF-G treatment in co-culture with endometrial cancer cells

Our qRT-PCR results indicate statistically significant alterations in the expression level of *IL6* in SF-G treated RAW264.7 cells during co-culture, compared with the control RAW264.7 cells (Fig. 3; $p < 0.05$). In turn, SF-G treatment has been demonstrated to induce inflammation under co-culture conditions. The expression of the CD86 and CD163 genes, significant indicators in macrophage polarization, also changed according to SF-G application. The expression of CD86, a pro-inflammatory marker, was significantly increased ($p < 0.05$) in macrophage cells that were co-cultured with cancer cells and treated with SF-G.

3.3 Expression of migration-related genes was altered in co-culture of endometrial cancer cells with SF-G-treated and non-treated macrophages

The migration ability of endometrial cancer cells co-cultured with macrophage cells was conducted by gene expression analysis. Based on the results acquired, the expression levels of vimentin (*VIM*) and twist family bHLH transcription factor 1 (*TWIST1*), which are key migration-related genes in cancer cells, were significantly increased in the co-culture with macrophages ($p < 0.05$). However, *VIM* and *TWIST1* expression was significantly decreased in Ishikawa cells co-cultured with SF-G-treated macrophages ($p < 0.05$). Furthermore, the decrease in *CDH1* and the increase in *CDH2*, which are epithelial-mesenchymal transition (EMT) markers, were found to be significant. In contrast to these results, it was observed that the migration of cancer cells was inhibited in the co-culture with SF-G-treated RAW264.7 cells (Fig. 4; $p < 0.05$).

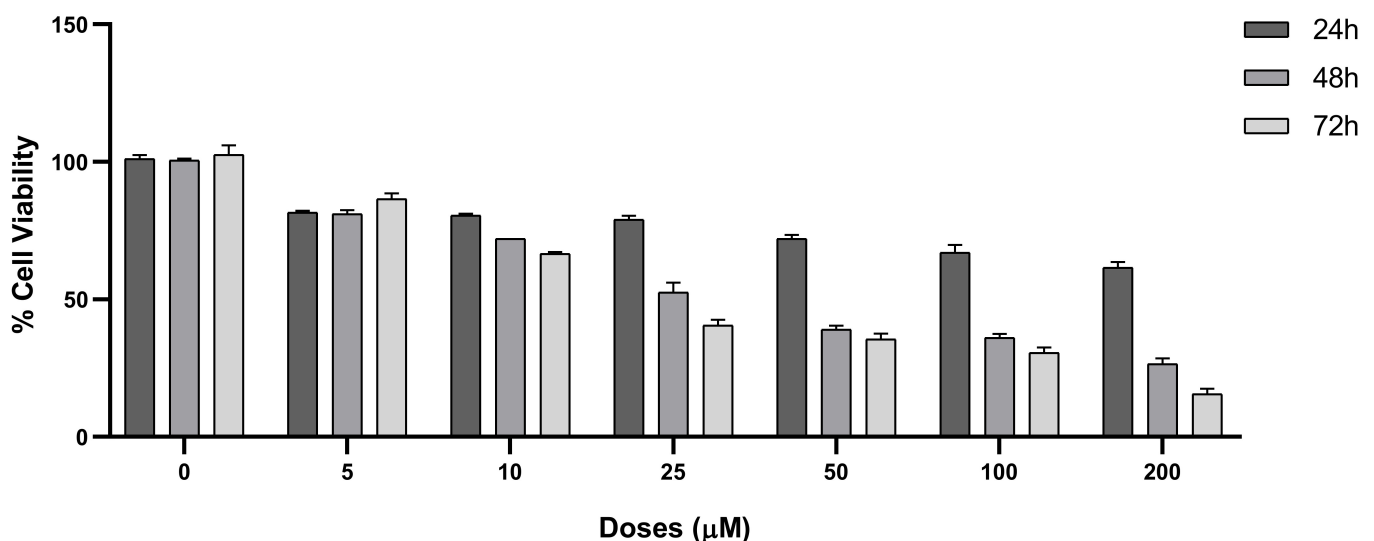


FIGURE 2. Cytotoxic effect of SF-G on RAW264.7 murine macrophage cells. CCK-8 test was used to assess growth inhibition on RAW264.7 cells after 24 h, 48 h, and 72 h treatment with SF-G doses ranging from 0–200 μM . All groups had three biological replicates.

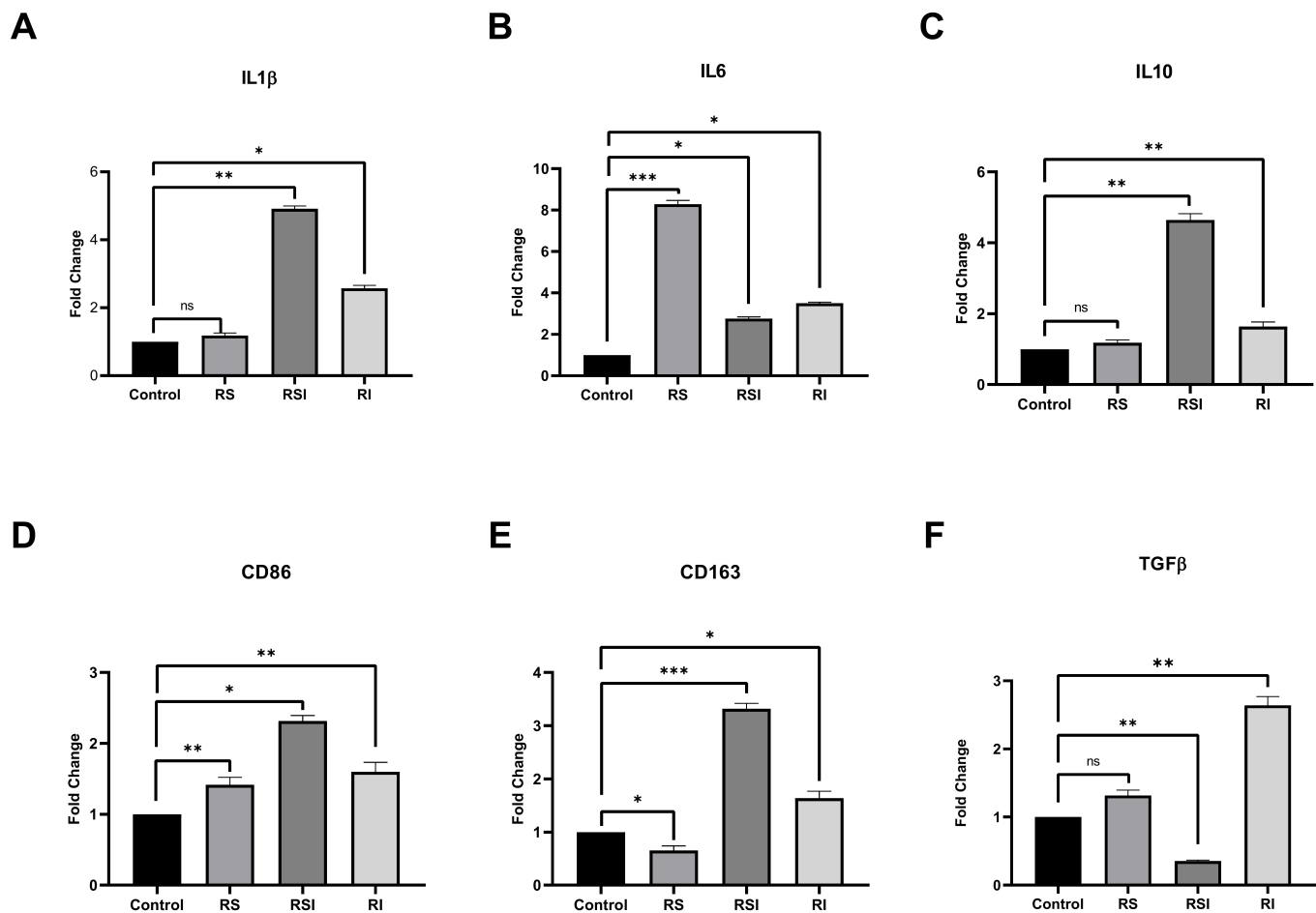


FIGURE 3. Expression changes of inflammation pathway-related genes. Effect of SF-G on IL1 β (A), IL6 (B), IL10 (C), CD86 (D), CD163 (E), and TGF β (F) mRNA levels in RAW264.7 cells after treatment with IC₂₀ for 24 hours. (Control, RAW264.7; RS, RAW264.7 + SF-G; RSI, RAW264.7 + SF-G + Ishikawa cells; RI, RAW264.7 + Ishikawa cells.) Target gene relative quantification was performed using 2^{- $\Delta\Delta C_t$} technique. The housekeeping gene in qRT-PCR studies was GAPDH. The fold changes in control and treatment groups were shown in bar graphs using GraphPad Prism software version 8.0.2 (California, USA). The data was examined using student's *t*-test. A *p*-value of < 0.05 was considered statistically significant. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. *p*-values > 0.05 were reported as statistically not significant (ns). All groups had three biological replicates. IL1 β , interleukin 1 beta; IL6, interleukin 6; IL10, interleukin 10; TGF β , transforming growth factor beta; CD86, cluster of differentiation 86; CD163, cluster of differentiation 163.

3.4 Cytokines protein levels of macrophage cells were changed w/wo SF-G treatment in co-culture with endometrial cancer cells

It has been shown that the level of IL1 β mRNA transcript increased significantly in co-culture with cancer cells group compared with control macrophage cells (Fig. 3A; *p* < 0.05). This result prompted an investigation into its reflection at the protein level. The ELISA results demonstrated that the level of IL1 β was significantly stimulated by SF-G co-cultured with cancer cells (Fig. 5; *p* < 0.05). Furthermore, the level of IL6 mRNA transcript has significantly increased in the co-culture with cancer cells group compared with control macrophage cells (Fig. 3B; *p* < 0.05).

According to the ELISA analysis results, the amounts of IL1 β and IL6 cytokines secreted by RAW264.7 cells with and without SF-G treatment when co-cultured with Ishikawa cells were similar to mRNA transcript levels (Fig. 3A,B).

3.5 Nitric oxide level of macrophage cells was changed w/wo SF-G treatment in co-culture with endometrial cancer cells

The amount of NO released from unstimulated RAW264.7 cells was significantly increased after co-culture with Ishikawa cells (*p* < 0.05). In contrast, NO release from SF-G-treated macrophages in the cancer-macrophage co-culture was significantly decreased compared with control macrophages (Fig. 6; *p* < 0.05).

4. Discussion

The role of macrophage polarization in inflammation [19], regeneration of tissues [20], and tumor niche [21] has been well-documented. The macrophages interact with other cells in the TME, including regulatory T cells, cytotoxic T lymphocytes, cancer associated fibroblasts (CAFs), and natural killer cells to mediate tumor cell destruction. Macrophages, referred

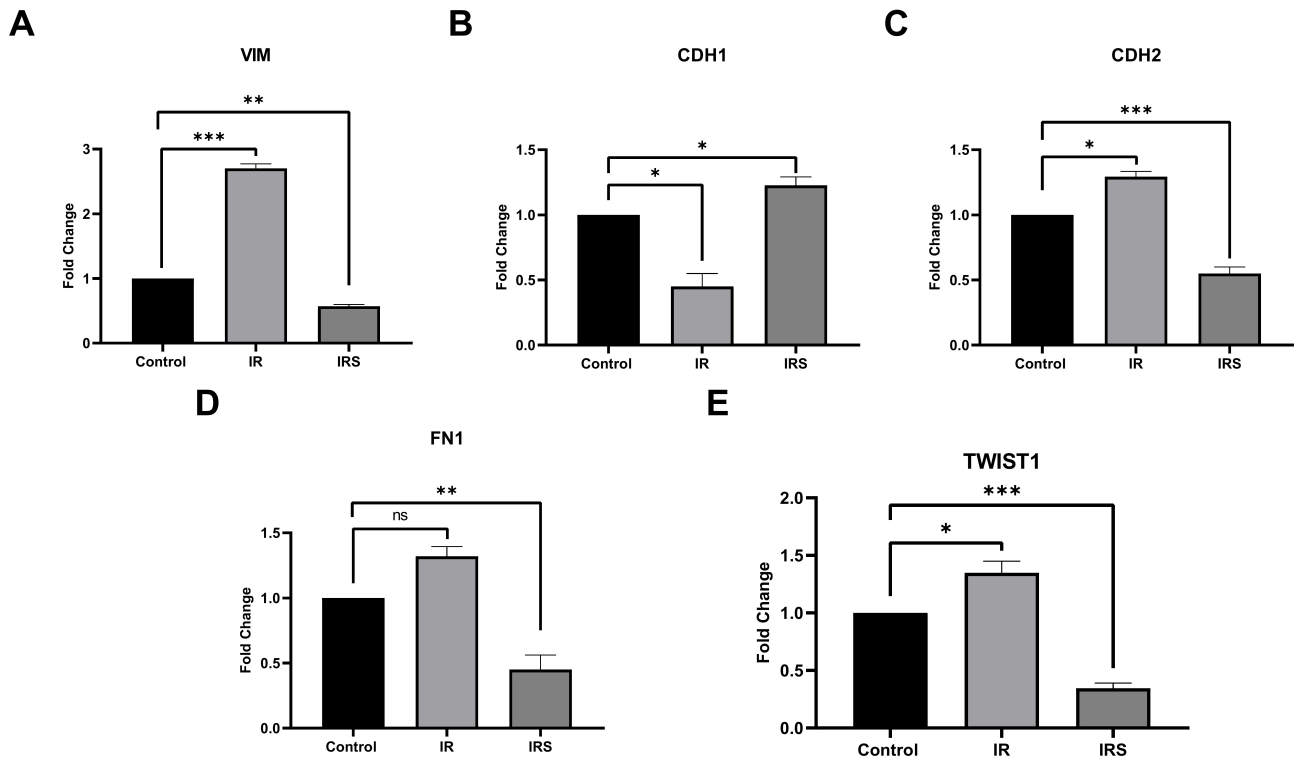


FIGURE 4. Expression changes of migration pathway-related genes in Ishikawa cancer cells. The mRNA levels of VIM (A), CDH1 (B), CDH2 (C), FN1 (D) and TWIST (E) in Ishikawa cells co-cultured with SF-G-treated and non-treated RAW264.7 (Control, Ishikawa cells; IR, Ishikawa cells + RAW264.7; IRS, Ishikawa cells + RAW264.7 + SF-G). Target gene relative quantification was performed using $2^{-\Delta\Delta C_t}$ technique. The housekeeping gene in qRT-PCR studies was GAPDH. The fold changes in control and treatment groups were shown in bar graphs using GraphPad Prism software version 8.0.2 (California, USA). The data were examined using student's *t*-test. A *p*-value of < 0.05 was considered statistically significant. **p* < 0.05 , ***p* < 0.01 and ****p* < 0.001 . *p*-values > 0.05 were reported as statistically not significant (ns). All groups had three biological replicates. VIM, vimentin; CDH1, cadherin 1 (E-cadherin); CDH2, cadherin 2 (N-cadherin); FN1, fibronectin 1; TWIST1, twist family bHLH transcription factor 1.

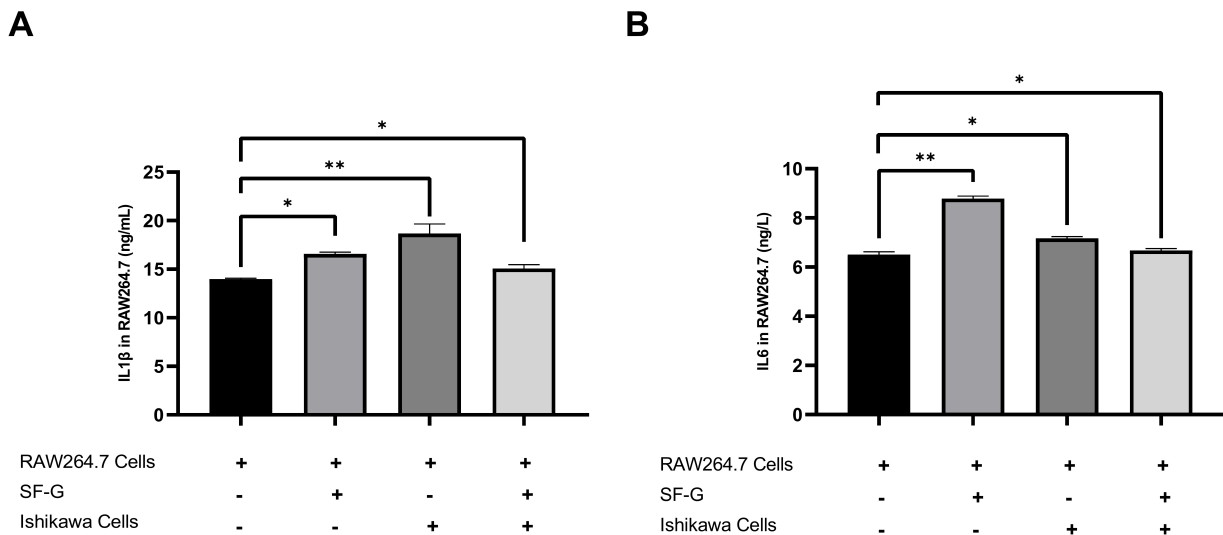


FIGURE 5. Amounts of IL1β and IL6 in RAW264.7 cells treated with SF-G. The levels of IL1β (A) and IL6 (B) calculated in the control RAW264.7 and SF-G-treated RAW264.7 cell line were depicted using GraphPad Prism (Version 8.0.2, GraphPad software Inc., San Diego California, USA). The data were analyzed using student's *t*-test. The results with *p*-values < 0.05 were considered as statistically significant. **p* < 0.05 and ***p* < 0.01 . All groups had three biological replicates. SF-G, sophoraflavanone G; IL1β, interleukin 1 beta; IL6, interleukin 6; (+) indicates the presence and (-) indicates the absence.

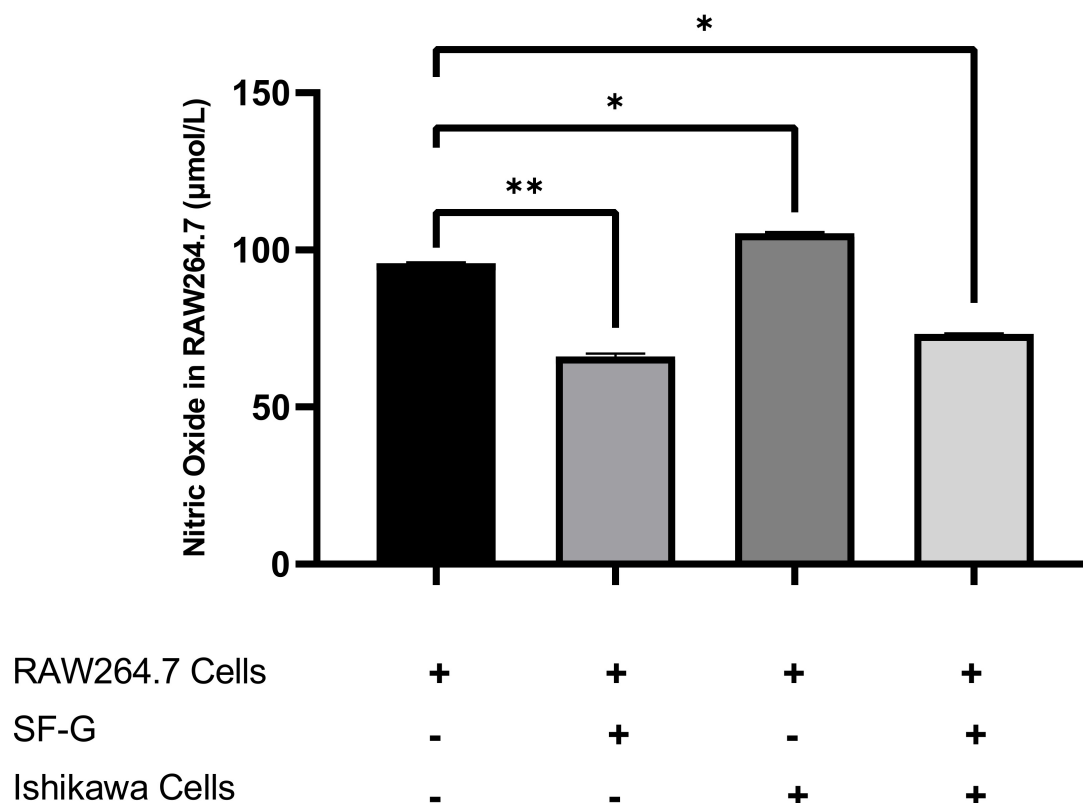


FIGURE 6. Amounts of nitric oxide (NO) in RAW264.7 cells. The levels of nitric oxide (NO) calculated in the control RAW264.7, SF-G-treated RAW264.7 and co-cultured with Ishikawa cells were depicted using GraphPad Prism software version 8.0.2 (California, USA). The data were analyzed using student's *t*-test. The results with *p*-values < 0.05 were considered as statistically significant. **p* < 0.05 and ***p* < 0.01. All groups had three biological replicates. SF-G, sophoraflavanone G; (+) indicates the presence and (-) indicates the absence.

to as TAMs, infiltrate the microenvironment of diverse solid tumors or invade tissues affected by malignancies. TAMs are a crucial component of the TME that affects chemoresistance, immune system modulation, tumor angiogenesis, metastasis, and tumor progression. According to a study, the increased TAM-inferred IL6 has a stronger effect on inflammatory responses, which advances the development of hepatocellular carcinoma and improves it as a liver cancer that uses Signal transducer and activator of transcription 3 (STAT3) signaling [22]. Due to their sensitive ability to introduce antigens, TAMs have a phenotype of M2-like macrophage and offer tangible assistance for tumor proliferation and metastasis. TAMs are primarily classified and identified based on their activity, which include immune system modulation, angiogenesis, and metastasis. For instance, the sequence of recognition of TAMs has been determined using CD206, CD163 and, B7-H4 [23]. This study demonstrated that *CD163* expression was significantly increased in the co-culture of RAW264.7 and Ishikawa cells. Therefore, macrophages co-cultured with cancer cells increased the expression of *CD163*, the M2 type marker (Fig. 3E). Moreover, it was found that *CD86* expression significantly increased in macrophage cells treated with SF-G, and in macrophage-cancer co-culture (Fig. 3D).

In this study, *CD163* expression level was significantly increased in the co-culture of RAW264.7 and Ishikawa cells, indicating that macrophages co-cultured with cancer cells exhibited an M2-type phenotype (Fig. 3E). Conversely,

CD86 expression was significantly increased in SF-G-treated macrophages, both in monoculture and in macrophage-cancer co-culture (Fig. 3D).

Macrophages release various proinflammatory cytokines, including IL1 β and IL6, and tumor necrosis factor-alpha (TNF- α), which may be involved in inflammation that promotes tumor growth. It may also boost the immune system, but it also encourages cancer cells to proliferate and survive. IL6 stimulates the Janus kinase (JAK)/STAT3 pathway, which leads to cell division, proliferation, and ultimately death [24]. Endothelial cells are stimulated by IL1 β to produce vascular endothelial growth factor (VEGF), which encourages angiogenesis and facilitates tumor invasion and spread. Additionally, it promotes the growth of tumors by stimulating the production of TNF- α , TGF β , and IL6 [25]. In our study, *IL6* expression increased significantly in macrophages treated with SF-G, while *IL1 β* expression showed an insignificant increase. In addition, in the co-culture of macrophages and cancer cells, IL1 β and IL6 increased significantly in the SF-G treated group. Kong *et al.* [26] discovered that TAM-produced IL6 triggered the growth of hepatocellular carcinoma (HCC) by activating the STAT3 pathway, indicating that IL-6 had a role in the development of HCC. In this study, we showed that significantly increasing the expression of *IL6* by RAW264.7 in the co-culture of macrophages and endometrial cancer cells caused a significant increase in migration-related genes (*VIM*, *CDH2*

and *TWIST1*) in Ishikawa cells, and our results (Fig. 4; $p < 0.05$) were consistent with the study of Wu and Zhou [27].

TGF β is produced by activated macrophages and exerts both pro- and anti-inflammatory effects [28]. In the early stages of tumor formation, TGF β can reduce cell cycle progression and induce apoptosis. However, TGF β also promotes epithelial-mesenchymal transition (EMT), facilitating tumor spread. Elevated TGF β levels can inhibit the anti-tumor T-cell response [29]. In our study, TGF β expression was significantly increased in macrophages co-cultured with cancer cells, whereas it was significantly decreased in macrophages in the SF-G-treated co-culture (Fig. 3F; $p < 0.05$). In macrophages treated with SF-G alone, TGF β levels increased slightly but not significantly. These results suggest that TGF β in the cancer co-culture is involved in promoting cancer progression, and SF-G treatment can modulate this effect.

M2 macrophages are generally immunosuppressive, produce IL10, and do not induce arginase or nitric oxide synthase 2 (NOS2), yet they play a key role in repressing M1 macrophages [30]. In our study, reflecting the anti-inflammatory role of IL10, its expression increased significantly-by approximately fivefold-in the co-culture with SF-G-treated macrophages. Additionally, IL10 expression was significantly increased in the macrophage-cancer co-culture compared with the control (Fig. 3C; $p < 0.05$). Results indicate the anti-inflammatory response is enhanced in SF-G-induced co-culture conditions.

Macrophages can create a mutagenic environment by releasing inflammatory mediators, such as TNF, interferon-gamma (IFN γ), IL6, reactive oxygen species (ROS), and nitrogen compounds, which may promote cancer development [31]. Ogger and colleagues investigated the mechanism of respiratory infections, and lung inflammation demonstrated that alveolar macrophages in mice produced increased ROS after mechanical ventilation [32]. To protect alveolar cells from stretch-induced damage or apoptosis *in vitro*, macrophages may produce NO in close proximity. Along with macrophages, lung surfactants regulate the cellular surface tension of alveolar cells.

In our study, NO release from macrophages increased during co-culture with cancer cells. However, NO levels decreased significantly in SF-G-treated macrophages and in the SF-G-treated macrophage-cancer co-culture (Fig. 6). Overall, these results indicate that SF-G reduces NO release in macrophages, both in monoculture and co-culture with cancer cells. This suggests that macrophages in co-culture conditions can promote a pro-inflammatory response while avoiding NO-related apoptosis.

5. Conclusions

Drugs used to eliminate cancer cells are directly toxic to cancer cells, but they also affect many other cells in the cancer tissue. Immune cells, especially those around the cancer, are affected by anti-cancer drugs. Immune cells are very sensitive cells. Macrophages are located in the cancer niche, as in many tissues. Cancer targeted drugs are also absorbed by macrophages surrounding the cancer. Macrophages create a strong two-way immune response; while supporting cancer progression,

they also ensure that cancer is eliminated. In this study, the effects of SF-G, which has been shown to have anti-cancer and inflammatory effects, on both macrophages and cancer cells in cancer co-culture were evaluated separately. SF-G supported the pro-inflammatory effect of macrophages applied with SF-G in co-culture but also suppressed the migration of cancer cells in co-culture.

While our results corroborate the proposed hypothesis in these models, validation on various cell lines for endometrial cancer and macrophages would further increase the generalizability of results. Further investigations using more cell models, comprising patient-derived cells and *in vivo* systems, will be necessary to confirm the translational value of our findings.

Our study may provide ideas for many future studies; investigating how other cells in the cancer niche are transformed by anti-cancer drugs may show new treatment targets.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

FSC—methodology; software; investigation; data curation; visualization; project administration. CEG—formal analysis; resources; supervision; funding acquisition. Both authors contributed to the conceptualization and validation of the study, and to the writing of the original draft as well as the review and editing of the manuscript. Both authors have read and agreed to the published version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study is an *in vitro* investigation and does not involve any primary human or animal tissues or cells. All cell lines used were commercially available; therefore, ethics committee approval was not required.

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Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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