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RNA Sequence Analysis Reveals Expected and Novel Immuno-Modulatory Activities by *Sutherlandia frutescens*

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Abstract

Sutherlandia frutescens (*S. frutescens*) has been traditionally used as an herbal medicine to ameliorate symptoms associated with cancer, infectious diseases, as well as inflammation. The objective of this investigation was to explore the impact of *S. frutescens* on the expression of genes in a murine macrophage cell line (i.e., RAW 264.7). We found that treatment with an ethanolic-extract of *S. frutescens* (SFE) 1 h prior to the stimulation with LPS and IFNγ for 24 h significantly affected the expression of 715 genes in RAW 264.7 cells. When the post-stimulation period was shortened to 8 h, the number of genes that were significantly impacted by SFE diminished to 50. Pathway analysis revealed that inflammatory signaling pathways, such as NF-κB, MAPK, and TNF, as well as signaling pathways associated with immune-related responses, were inhibited by SFE treatment. These findings are consistent with previously reported anti-inflammatory activity of SFE and enable better understanding of the immune-modulating properties of this botanical. To our knowledge, this represents the first report on the impact of *S. frutescens* on global gene expression in an immune cell population.

Key Words: *Sutherlandia frutescens*, RNA sequencing, inflammation, TNF

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Introduction

Sutherlandia frutescens (*S. frutescens*) is a well-known medicinal plant widely used in southern Africa[1]. Previous reports indicated that this plant can be used for the treatment of different diseases, such as cancer, inflammation, and infectious diseases[2-5]. The mechanisms for these putative medicinal properties of *S. frutescens* have not been investigated extensively. We and others have reported that ethanolic and aqueous extracts of this plant possess anti-inflammatory and immuno-stimulatory activities, respectively[6,7]. However, the mechanism(s) by which *S. frutescens* may modulate health outcomes remains unclear. Recently, our group demonstrated that an ethanol extract of *S. frutescens* down-regulated 50% of Hedgehog-responsive genes in murine prostate cancer cells (i.e., TRAMPC2)[6]. Also, we have previously reported that extracts from *Sutherlandia* can diminish NF-κB, ERK, AP-1, and JAK-STAT1 signaling in macrophages and microglia cells [8-11].

Macrophages play important roles in both innate and adaptive immune responses, including host defense, wound healing, and immune regulation[12]. Macrophages can recognize pathogen-associated molecular patterns (PAMP) and other immune stimuli via receptors, such as toll-like receptors (TLR)[13]. After binding to the TLR receptors, several classical signaling pathways, including NF-κB, MAPK, and JAK-STAT are activated, resulting in the production of nitric oxide (NO), reactive oxygen species (ROS), and inflammatory cytokines/chemokines[14,15]. Cytokines and other mediators subsequently modulate systemic responses to inflammation, cancer, and other disease states[16,17]. In light of our previous results indicating that extracts of *Sutherlandia* could significantly reduce signaling through a number of key immune/inflammatory pathways, we sought to

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explore the effect of *S. frutescens* on global gene expression in macrophages using RNA sequencing (RNA-Seq).

RNA-Seq has been widely used in biomedical studies to quantify global transcript concentrations using high-throughput sequencing technologies[18]. The resulting large sets of transcript data can then be used for a deeper understanding of immune responses and provide new strategies for clinical treatment[19]. Because of their central role in regulating immune responses and involvement in inflammatory conditions, the scientific literature contains dozens of reports of global gene expression in macrophages following exposure to pathogens, pathogenic components, or immune mediators[20]. The source of macrophages used for "gene array" research has varied from human peripheral blood monocyte derived macrophages and bone marrow derived or peritoneal derived macrophages from mice, or macrophage-like cell lines of human (e.g., THP-1, U937) or murine (e.g., RAW 264.7, J774, PU5-1.8) origin. For this study, we used the murine macrophage cell line, RAW 264.7 cells. We examined the immune-modulatory activity of *S. frutescens* in these cells with and without co-stimulation of LPS and IFNγ. The use of LPS and IFNγ together results in what is referred to as classically activated (or M1) macrophages[12].

Results

S. frutescens treatment affected gene expression in RAW 264.7 cells in a concentration and time-dependent manner

As shown in Table 1 and Fig. 1, acute treatment with low dose of SFE (8 μg/mL) showed no effect on gene expression. Meanwhile, only 2 genes (i.e., *Oasl2* and *Slfn5*)

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were down-regulated and 2 genes (i.e., *Ptgs1* and *BC005764*) were up-regulated after treated with 8 μg/mL SFE for 24 h. In contrast, 80 μg/mL of SFE exhibited a stronger impact on gene expression. Cells exposed to 80 μg/mL SFE for 8 h had 79 differentially expressed genes (22 down-regulated and 57 up-regulated) compared to untreated cells. A total of 226 genes (123 were down-regulated and 103 were up-regulated) were modulated by the treatment of 80 μg/mL SFE for 24 h in the RAW 264.7 cells (Table 1 and Supplemental Table 1). Consequently, SFE shows both time- and concentration-dependent impacts on the number of DEGs.

Table 1. Numbers of differentially expressed genes in murine macrophages (RAW 264.7) following treatment with the ethanol extract of *S. frutescens* with or without co-stimulation with LPS (10 ng/mL) and IFNγ (0.1 ng/mL).

Time	Treatment	Total	Down-regulated	Up-regulated
8 h	Unstim Ctrl vs Stim Ctrl	2803	1312	1491
8 h	Unstim Ctrl vs 8 μg SFE	0	0	0
8 h	Unstim Ctrl vs 80 μg SFE	79	22	57
8 h	Stim Ctrl vs 8 μg SFE	0	0	0
8 h	Stim Ctrl vs 80 μg SFE	50	28	22
24 h	Unstim Ctrl vs Stim Ctrl	4319	2350	1969
24 h	Unstim Ctrl vs 8 μg SFE	4	2	2
24 h	Unstim Ctrl vs 80 μg SFE	226	123	103
24 h	Stim Ctrl vs 8 μg SFE	2	0	2
24 h	Stim Ctrl vs 80 μg SFE	715	226	449

The gene expression values are from two biological replicates of different experiment conditions. The differentially expressed genes are defined as $p < 0.05$, false discovery

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rate < 0.05, fold change greater than 2. Unstim: Unstimulated group; Stim: Stimulated group; Ctrl: Control; SFE: Ethanolic extract of *S. frutescens*.

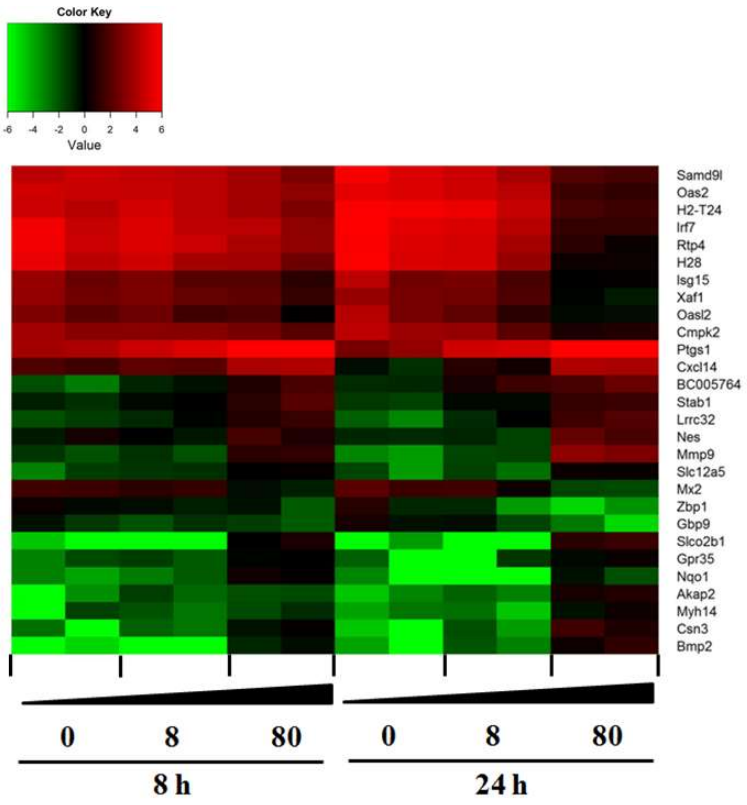

Fig. 1. Differentially expressed genes (DEG) affected by ethanol extract of S. frutescens (SFE) in LPS/IFNγ co-stimulated murine macrophages, RAW 264.7 cells. Gene expression values are represented by Log2 transformed normalized RNA-Seq reads. The figure is showing the gene expression values from 2 biological replicates of RAW 264.7 cells in different conditions as indicated in the diagram. Genes shown in this diagram are the top 30 (by fold change) genes that are either up-regulated (red) or down-regulated (green). Black means no change compared to the control (cells without SFE

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treatment). The gene changes in the treatment with "0" SFE shows these genes affected by co-stimulation with LPS and IFNγ compared to unstimulated cells.

S. frutescens co-treatment altered the response of RAW264.7 cells to LPS and IFNγ

Co-stimulation with LPS and IFNγ for 8 h affected a total of 2803 genes of which 1312 were down-regulated and 1491 were up-regulated (Table 1 and Supplemental Table 2). Stimulation for 24 h modulated 4319 genes in RAW 264.7 cells (2350 down-regulated and 1969 up-regulated) as compared to cells without stimulation. Gene expression was not affected by co-treatment with 8 μg/mL SFE for 8 h in RAW 264.7 cells, while the expression of 2 genes (i.e., *Rpph1* and *Rmrp*) was increased by treatment with this concentration of SFE for 24 h. Treatment with 80 μg/mL SFE 1 h prior to the stimulation of LPS/IFNγ for 8 h led to 50 genes affected by SFE, including 28 down-regulated and 22 up-regulated as compared to cells treated with stimuli only (Table 1 and Fig. 1). Meanwhile, treatment with 80 μg/mL SFE 1 h prior to the stimulation of LPS/IFNγ for 24 h resulted in a total of 715 genes altered of which 266 were down-regulated and 449 were up-regulated (Table 1 and Supplemental Table 3).

The function of differentially expressed genes affected by S. frutescens

To evaluate the function of differentially expressed genes affected by SFE, functional annotation analysis was performed using DAVID[21]. Since treatment with 8 μg/mL SFE showed a limited impact on gene expression in all conditions and their functions were not enriched (data not shown), the functional annotation was mainly focused on the DEGs altered by treatment with 80 μg/mL SFE. Without LPS and IFNγ stimulation, differential expressed genes were modulated by the treatment of SFE mainly

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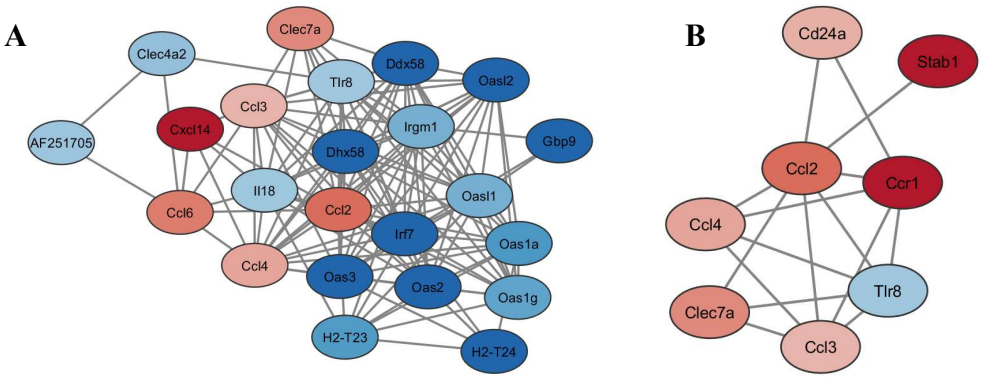
involved in signaling pathways associated with immune and inflammatory responses (Fig. 2A, 2B, 2C, and 2D), cytokine and cytokine receptor interaction (Fig. 2E), chemokine signaling pathway (Fig. 2F), and numerous other signaling pathways as listed in Supplemental Table 4. As summarized in Fig. 2G, SFE reduced the expression of Tlr3, Tlr8, Ifih1, and Ddx58, which induced the reduction of Irf7, an important regulator of type I interferon. Treatment of SFE also reduced the expression of genes in STAT1 signaling, such as Stat1, Irf9. The SFE also increased the expression of several genes, including Cxcl14, Bmp2, Ccr1, and Stab1. Cxcl14 is known as a tumor suppressor in various types of cancer, such as head and neck cancer, breast cancer, lung cancer, and hepatocellular carcinoma [22-26]. To simplify the changes of genes related with immune function under the un-stimulated condition, we propose the TLR and JAK-STAT signaling pathways by which SFE can affect anti-viral and anti-cancer activities under the un-stimulated condition (Fig. 2G). With co-stimulation of SFE with LPS and IFNγ, analysis revealed that DEGs affected by SFE were involved in numerous intracellular signaling pathways at 8 h and 24 h signaling involved in-infectious diseases and immune responses (Supplemental Table 5).



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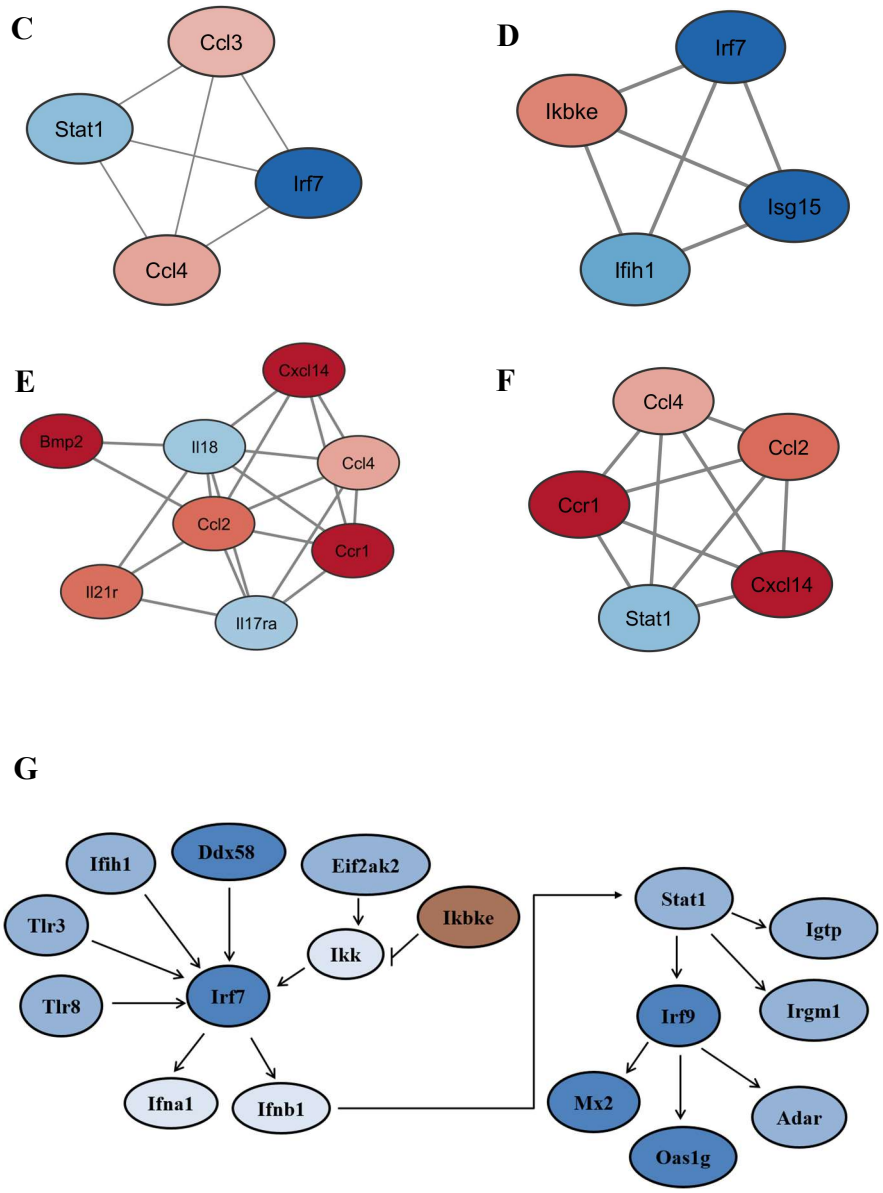

Fig. 2. Ethanol extract of *S. frutescens* modulated genes associated with immune function related signaling pathway in unstimulated RAW 264.7 cells. Cells were treated with 80 μg/mL ethanol extract of *S. frutescens* for 24 h, and cell lysates were subsequently harvested for RNA sequencing. The differentially expressed genes (DEGs) were identified by the edgeR program, and the gene ontology analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Further, the impact of *S. frutescens* on the signaling pathways was analyzed using Kyoto

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Encyclopedia of Genes and Genomes (KEGG). The gene interaction networks associated with immune responses **(A)**, inflammatory responses **(B)**, toll-like receptor signaling pathway **(C)**, RIG-like receptor signaling pathway **(D)**, cytokine-cytokine receptor interaction **(E)**, and chemokine signaling pathway **(F)** were visualized by Cytoscape. The signaling pathway associated with viral infection was summarized in **(G)**. The color light blue indicates the genes that were reduced 2-4 fold and the dark blue indicates the genes that were reduced greater than 4 fold by treatment of SEF. The color light red indicates the genes that were increased 2-4 fold and the dark red indicates the genes that were increased greater than 4 fold by treatment of SEF. Irf: Interferon regulatory factor, Ifih1: IFN induced with helicase C domain 1, Ddx58: DEAD (Asp-Glu-Ala-Asp) box polypeptide 58, Ikbke: I-kappa-B kinase epsilon, IKK: I-Kappa B kinase, Eif2ak2, Eukaryotic translation initiation factor 2-alpha kinase 2, Oas1g: Oligoadenylate synthetase 1G, Adar: Adenosine deaminase, Mx: MX dynamin-like GTPase, Irgm1: Immunity-related GTPase family Member 1, Igtp: Interferon gamma induced GTPase, Ccl: Chemokine (C-C motif) ligand, Il: Interleukin, H2-H24: Histocompatibility 2, T region locus 24, Clec: C-type lectin domain, Cxcl: Chemokine (C-X-C motif) ligand, Oas: 2'-5' oligoadenylate synthetase, Oasl: 2'-5' oligoadenylate synthetase-like, Ccr: Chemokine (C-C motif) receptor, Gbp: Guanylate-binding protein 9, Isg: ISG15 ubiquitin-like modifier.

In this study, we also determined the impact of SFE treatment on immune related genes in RAW 264.7 cells under un-stimulated or stimulated with LPS/IFNγ. Treatment with 80 μg/mL SFE alone for 8 h altered the expression of 18 genes related to immune

 doi:10.20944/preprints201809.0406.v1

and inflammatory responses, while 39 genes were regulated after treatment of SFE for 24 h (Supplemental Table 6). Upon stimulation with LPS/IFNγ, exposure to SFE for 8 h inhibited the expression of *Ifnb1* and *Nod1*, which are highly associated with inflammatory responses; however, SFE also increased the expression of *Il1a* and *Cxcl14* which are pro-inflammatory factors. Among the 715 differentially expressed genes affected by the 24 h treatment with SFE, 117 of these genes were associated with immune function (Supplemental Table 7).

Analysis of the pathways and networks of immune-related genes affected by *S. frutescens* with or without co-stimulation of LPS/IFNγ was performed using the KEGG database. SFE (80 μg/mL) altered numerous signaling pathways associated with immune function in RAW 264.7 cells with or without co-stimulation with LPS and IFNγ. This analysis clearly shows that the ethanol extract of *S. frutescens* (80 μg/mL) alone or co-treated with LPS/IFNγ modulated signaling pathways of cytokine-cytokine receptor interaction, cancer, and TNF in RAW 264.7 cells. The TLR, influenza A, herpes simplex infection, and endocytosis signaling pathways were affected by SFE at both 8-h and 24-h post-stimulation (Supplemental Figure 1). Expression of genes involved in the signaling pathways of cytokine-cytokine receptor interaction and TNF was also altered after exposure to 80 μg/mL SFE for 24 h (Fig. 3).

The present study also shows that the signaling pathways involved in cancer and rheumatoid arthritis in cells were diminished after treatment with 80 μg/mL SFE for 24 h. Ethanol extract of *S. frutescens* (SFE) altered the expression of genes associated with several infectious diseases, such as: tuberculosis, hepatitis B, hepatitis C, and Chagas disease.

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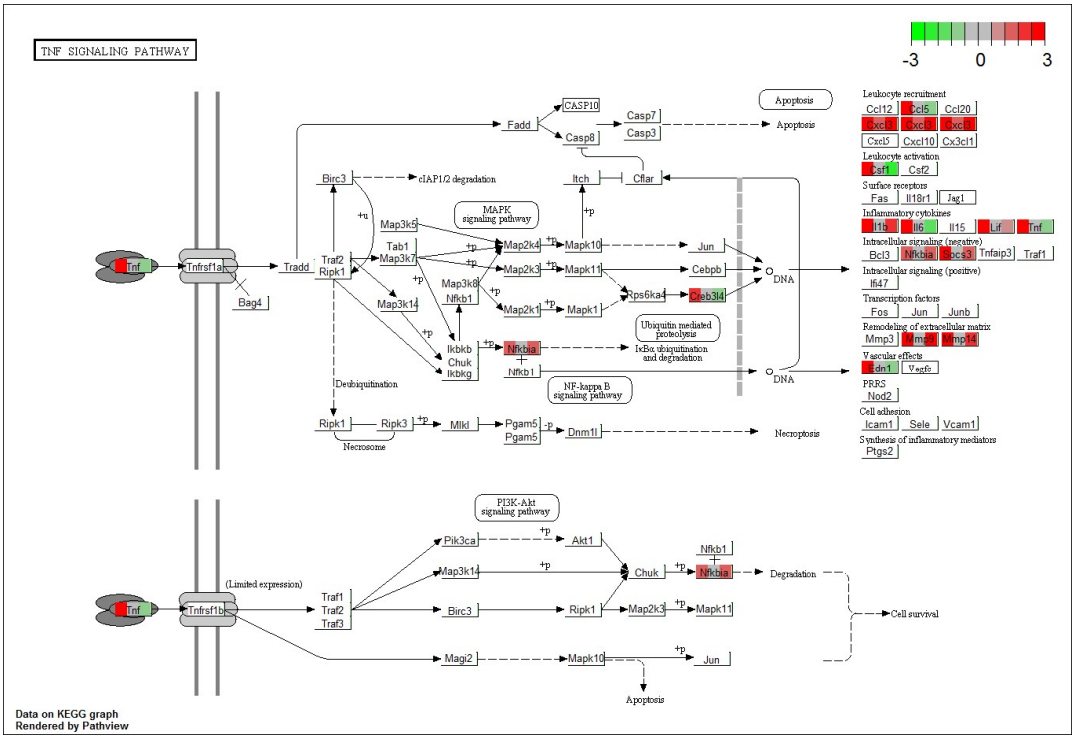

Fig. 3. Ethanol extract of *S. frutescens* (SFE) affects the cytokine-cytokine receptor interaction and TNF signaling in murine macrophages co-stimulated with LPS/IFNγ. Cells were treated with 80 μg/mL ethanol extract of *S. frutescens* 1 h prior to co-stimulation with LPS (10 ng/mL) and IFNγ (0.1 ng/mL) for 24 h, and cell lysates were subsequently harvested for RNA sequencing. The differentially expressed genes (DEGs) were identified by the edgeR program, and the impact of *S. frutescens* on the signaling pathways was analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG). In these diagrams, genes highlighted with colors are affected by SFE. In the box of affected genes, the analysis results were shown from three different comparisons (from left to right): Unstimulated control vs stimulated control, stimulated control vs stimulated + 8 μg/mL SFE, and stimulated vs stimulated + 80 μg/mL SFE. The color represents expression value; the differences of colors represent alteration.

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Functional analysis of DEGs induced by LPS/IFNγ stimulation but reversed by treatment of SFE

GeneVenn diagram analysis showed that 7 out of 1312 genes which are down-regulated by co-stimulation of LPS/IFNγ are increased by treatment of 80 μg/mL SFE for 8 h, and 19 out of 1491 genes up-regulated by the stimulation are suppressed by the treatment of SFE (Supplemental Fig. 2). In the 715 DEGs affected by 80 μg/mL SFE for 24 h, 268 of were reversed by the stimulation of LPS/IFNγ (Fig. 4A). Following analysis revealed these genes that are mainly involved in the immune and inflammatory responses, and regulating MAPK activities (Fig. 4 B-E). The KEGG analysis demonstrates that these genes were associated with PI3K-AKT signaling, cytokine-cytokine receptor interaction, TLR, NOD-like receptor, MAPK, TNF, PPAR, RAS, RAP1, HTLV-I infection, and Herpes simplex infection.

As shown in Fig. 4, SFE significantly reversed the effects induced by LPS/IFNγ and those genes that are down-regulated are presented in green while red represents the up-regulated genes. The impact of SFE treatment on the signaling pathways associated with inflammatory responses is summarized in Fig. 4F. SFE modulated the genes associated with NF-κB and MAPK signaling pathways, leading to a potential immune response change during LPS/IFNγ treatment. SFE increases the expression of inhibitors or decreases the activators of NF-κB and MAPK signaling pathways. These effects led to a reduction in the expression of several pro-inflammatory mediators, such as TNF and IL6. However, the treatment of SFE also increased the expression of IL1α and IL1β.

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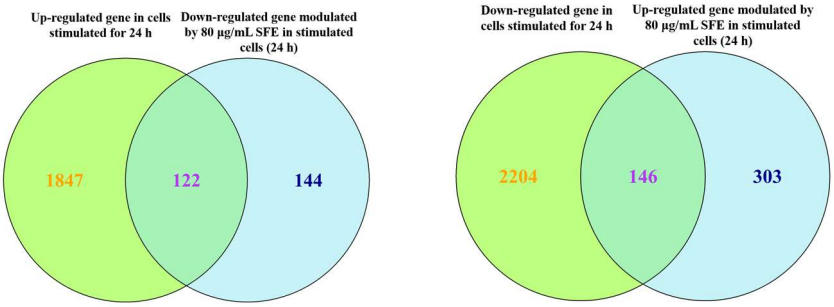

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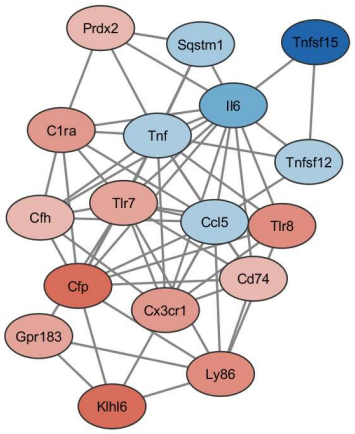

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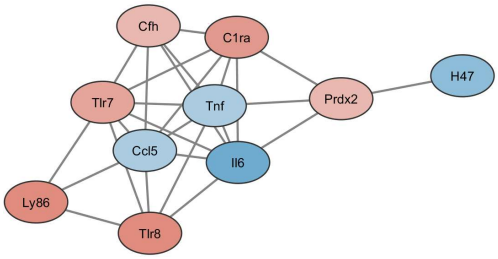

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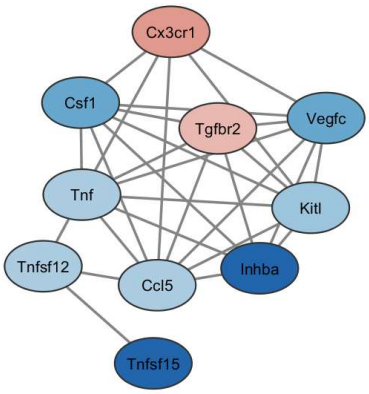

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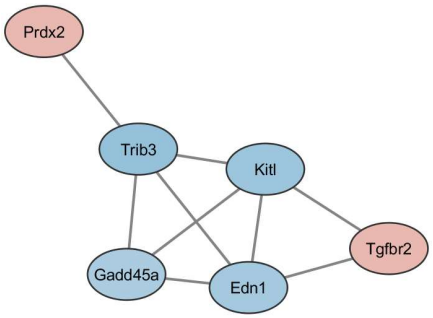

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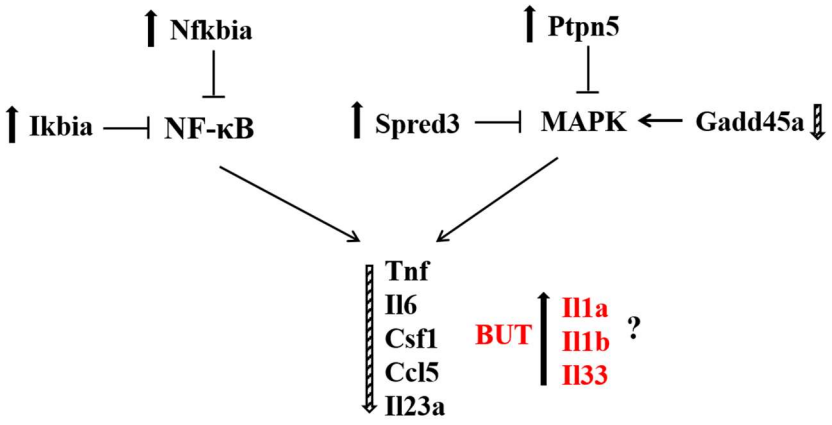

Fig. 4. Venn diagram and gene ontology of the genes affected by the ethanol extract of *S. frutescens* (SFE) and co-stimulation with LPS/IFNγ for 24 h. (A) Genes either up-regulated or down-regulated by co-stimulation of LPS/IFNγ with 80 μg/mL SFE for 24 h were submitted to edgeR for overlap analysis, and to generate the Venn diagrams. (B-E) The gene ontology was analyzed using the database of DAVID, the interaction of DEGs associated with immune response, inflammatory response, cytokine-cytokine receptor interaction, and regulation of protein kinase is shown. (F) Summary of the modulation of 24-h treatment with the SFE on NF-κB and MAPK signaling pathways in LPS/IFNγ co-stimulated RAW 264.7 cells. The color blue or symbol ↓ indicates the genes that were down-regulated genes while red or ↑ is for genes that were up-regulated. Ikbia: I-kappa-B inhibitor alpha, Nfkbia: Nuclear Factor- Kappa B inhibitor alpha, Ptpn5: Protein tyrosine phosphatase, non-receptor type 5, Spred3: Sprouty-related, EVH1 domain containing 3, Gadd45a: growth arrest and DNA-damage-inducible 45 alpha, Csf1: Colony stimulating factor 1, Edn1: endothelin 1, Tgfbr2: transforming growth factor, beta receptor II, Trib3: tribbles pseudokinase 3, Kitl: kit ligand, Prdx2: peroxiredoxin 2, Cd74: HLA class II histocompatibility antigen gamma chain, Ly86: lymphocyte antigen 86, Klhl6: kelch-like 6, Cx3cr1: chemokine (C-X3-C motif) receptor

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1, Gpr183: G protein-coupled receptor 183, Cfp: complement factor properdin, Cfh: complement component factor h, Tnfsf12: TNF superfamily, member 12, C1ra: complement component 1, r subcomponent A, Sqstm1: sequestosome 1, Inhba: inhibin beta-A, Tgfbr2: transforming growth factor, beta receptor II, Vegfc: vascular endothelial growth factor C.

Discussion

In our study, co-treatment of the murine macrophage cell line (RAW 264.7 cells) with LPS and IFNγ resulted in over 2800 differentially-expressed genes (DEGs) after just 8 hours, which increased to more than 4300 DEGs at 24 hours post-stimulation. Several other groups have reported global gene expression profiles of classically activated macrophages and our findings are consistent with those prior reports[20]. Not surprisingly, the majority of these genes are associated with the immune-related responses, including: inflammation, wound healing, and destruction of infectious agents.

The primary objective of the present study was to use RNA sequencing analysis to investigate the effects of an ethanol extract of *S. frutescens* (SFE) on global gene expression in murine macrophages at rest and during immune activation. Importantly, we observed that treatment with SFE significantly diminished the expression of genes involved in signaling pathways associated with immune responses, such as: NF-κB, MAPK, and JAK-STAT. These findings are in agreement with our previous studies[7,8] In this study, our use of RNA-seq provided insights into some of the possible molecular mechanisms through which *Sutherlandia* modulates macrophage function. For example, the treatment of SFE increased the expression of *Nfkbia* and *Ikbke*, which are two inhibitors of the NF-κB pathway. Similar to NF-κB, SFE modulated the expression of

 doi:10.20944/preprints201809.0406.v1

Spred-3, *Ptpn5*, and *Socs3* to reduce the activation of MAPKs and JAK-STAT. In addition to influencing these inflammatory signaling pathways, SFE was also able to modulate the signaling pathways of NOD-like receptor, p53, PI3K-AKT, and peroxisome proliferator-activated receptors (PPAR). It is through regulating these signaling pathways, that *S. frutescens* appears to reduce the expression of a number of pro-inflammatory genes (e.g., TNF) that are known to play critical roles in immune responses against infectious disease as well as in numerous inflammatory conditions.

The ability of SFE to reduce TNF-α expression provides a plausible explanation for several of the putative medicinal properties associated with *S. frutescens* use. As a pro-inflammatory cytokine, TNF-α is widely involved in several chronic inflammatory diseases, including rheumatoid arthritis, through modulating the production of inflammatory cytokines (i.e. IL-6) and growth factors (CSF-1)[27]. SFE treatment reduces the production of TNF both in murine macrophages and in mice[28]. The reduction of TNF by *S. frutescens* treatment may, in part, explain the medicinal activity of this herb on rheumatoid arthritis as well as insulin resistance in type 2 diabetes. While others[29-31] have reported that *S. frutescens* has a beneficial impact on insulin function, much is to be learned about how this botanical acts on insulin responsiveness. TNF modulates the expression of Smad3 in the TGF-β signaling pathway which is one of the critical anti-diabetes pathways[32]. We propose that the reduction in TNF production and subsequent signaling may play a critical role in the anti-diabetic activity of *S. frutescens*.

While there may be several health benefits associated with reduced production of TNF, there is evidence that *Sutherlandia* use could be problematic in some circumstances. TNF is known to play an important role in controlling tuberculosis[11,33],

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which may explain why treatment with *S. frutescens* increased the risk of tuberculosis infection in a recent clinical trial[11,34]. Since, TNF production is increased during many other infectious diseases, such as: pertussis, African trypanosomiasis, amoebiasis, hepatitis B, and influenza A, it remains to be determined if *Sutherlandia* use in individuals with any of these infections might also be contraindicated.

In conclusion, this is the first report investigating the effects of an ethanol extract of *S. frutescens* on global gene expression in the murine macrophage cell line, RAW 264.7. This study revealed that *S. frutescens* modulated numerous genes associated with signaling pathways which are linked to immune and inflammatory responses. Our data are consistent with the conclusion that the impact of SFE on TNF production can explain several medicinal properties of *S. frutescens*.

Materials and methods

Reagents

LPS (from *E. coli* 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine IFNγ was purchased from R&D Systems (Minneapolis, MN, USA). The fetal bovine serum (FBS) was purchased from Thermo Scientific (Logan, Utah, USA). RNeasy Mini Kit (Cat. No. 74104) was purchased from Qiagen (Valencia, CA, USA).

Preparation of ethanol extract of S. frutescens

The powder of *S. frutescens* (L.) R. Br. was purchased from Big Tree Nutraceutical (Fish Hoek, South Africa) and verified as described previously[10]. The ethanol extract of

 doi:10.20944/preprints201809.0406.v1

S. frutescens (SFE) was prepared, and the dry matter concentration was determined as described previously[7]. SFE was dried at 55°C under a vacuum (CentriVap Concentrater, Labconco, Kansas City, MO, USA), and then the dried SFE was re-suspended in DMSO with the final concentration of 84 mg/mL.

Cell culture and treatment

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM (Gibco, Grand Island, NY, USA) with 5% FBS, at 37°C with 5% CO_2. For all experiments, RAW 264.7 cells were seeded in 6-well plates, and cultured overnight in DMEM/5% FBS to reach > 90% confluency. Cells were pre-treated with or without SFE (8 or 80 μg/mL) for 1 h, then co-stimulated with LPS (10 ng/mL) and IFNγ (0.1 ng/mL) for 8 or 24 h. The cell lysates were then harvested for RNA extraction.

RNA extraction

Total RNA was extracted using the RNeasy Mini Kit following the manufacturer's instructions. Briefly, cell lysates were transferred into micro-centrifuge tubes with 600 μL of Buffer RLT. The RAW cell lysates were centrifuged at 13,000 rpm for 3 min, and the supernatant (~500 μL/sample) transferred into a new micro-centrifuge tube. The RNA was precipitated by adding the same amount of 70% ethanol, and harvested using the column provided in the kit. The concentration and quality of total RNA was determined using a Nano-Drop spectrophotometer (Thermo Science, Wilmington, DE, USA) and adjusted to 100 ng/mL by adding RNase-free water.

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RNA-Seq

A total of 2.5 µg of total RNA was sent to MU DNA core for RNA-Seq analysis. The cDNA sequencing libraries were generated from poly-A selected RNA using a TrueSeq library preparation kit (Illumina, Inc., San Diego, CA, USA). RNA-Seq was performed using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA). Using FASTX-Toolkit, the adaptor sequences were trimmed, low quality base call at the end of sequencing reads were subsequently clipped, and low quality sequencing reads were filtered. Filtered sequencing reads were aligned to mouse reference genomes mm9 (UCSC) using Tophat2[35,36]. Genes with less than one count per million were considered to be low expressed genes and were excluded from further analysis.

Differentially Expressed Gene analysis using edgeR

Only reads that were mapped to a unique location in the genome were retained to calculate gene expression value. The gene expression values (counts) were calculated using Multicom-Map[37]. Differentially Expressed Genes (DEGs) were analyzed using R/Bioconductor package edgeR [38]. The Log fold change (LogFC) was calculated using the average of library size-normalized read counts from replicates in the same treatment group. Genes with a false discovery rate (FDR) < 0.05[39], and LogFC ≥ 1 or ≤ -1 were defined as DEGs.

The heat maps were generated using function of "heatmap.2" through the 'gplot' package in R/Bioconductor package edgeR. Normalized gene expression values were Log_2 transformed, and loaded into the program to create the heat maps. The color

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represents expression value, and differences in color represent differences in gene expression.

Venn diagrams were generated using the VennDiagram package in R program[40].

Analysis of differentially expressed genes by edgeR and DAVID

The gene ontology analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[21]. Briefly, the gene symbol of DEGs between different treatments was loaded in the online interface for enrichment analysis. An enrichment score of 0.05 was used as a cutoff value to determine the over-represented gene ontology terms.

Signaling pathway analysis by Kyoto Encyclopedia of Genes and Genomes (KEGG)

The KEGG pathway analysis was conducted to investigate the impact of *S. frutescens* on signaling pathways[41]. The protein sequences of differentially expressed gene products were imported to the KEGG Automatic Annotation Server (KAAS) in the KEGG database for pathway prediction[42]. The KEGG orthology (KO)-gene relationships constructed were produced according to the KEGG database. The final pathways were visualized using the "Pathview" program based on the KO-gene-assignment file and fold change value for each gene under multiple comparisons[43]. The degree of log2 fold changes was highlighted with different colors.

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Protein-protein Interaction network construction

The functional clusters associated with immune responses from DAVID analysis were used for the gene network analysis to infer the gene interaction. The protein-protein association data for *Mus musculus* were downloaded from string database[44]. Then the gene interaction network for each function category was visualized by Cytoscape[45].

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Author contributions

Conceptualization, W.L., G.Y.S., D.B.L., W.R.F., and K.L.F.; Methodology, W.L., J.C., and K.L.F.; Validation, G.Y.S., D.B.L., W.R.F., and K.L.F.; Formal Analysis, W.L., Y.L., J.H., and C.C.; Investigation, W.L., Y.L., and J.D.B.; Resources, G.Y.S., D.B.L., W.R.F., J.C., and K.L.F.; Data Curation, W.L., and K.L.F.; Writing – Original Draft Preparation, W.L. and K.L.F.; Writing – Review & Editing, W.L., G.Y.S., D.B.L., W.R.F., and K.L.F.; Supervision, K.L.F.; Funding Acquisition, G.Y.S., D.B.L., and K.L.F.

Competing interests

The authors declare no conflict of interest.

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