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Tocotrienol-rich Fraction Modulated Genes Responsible for Inflammation in Lipopolysaccharide-stimulated RAW 264.7 Macrophages

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Inflammation plays a vital role in the pathogenesis of chronic non-communicable diseases (NCDs), the leading health issue worldwide. An earlier study reported that tocotrienol-rich fraction (TRF) showed better anti-inflammation effects in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages.
Aim: This study aimed to investigate the anti-inflammatory effects of tocotrienol-rich fraction at the molecular level by looking at the genes that were differentially regulated and pathways affected in LPS-stimulated macrophages exposed to TRF using the microarray approach.
Methods: A microarray study was carried out in LPS-stimulated RAW 264.7 macrophages. Total

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ribonucleic acid (RNA) was extracted from the RAW 264.7 cells treated with TRF (10µg/mL), alpha-tocopherol (10 µg/mL) or LPS (10 ng/mL). Untreated cells served as control. Enrichment analyses, such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG), were conducted for genes listed in the differentially expressed genes (DEGs).
Results: The microarray analysis showed that the expression of five genes [*Hamp, Interleukin-1a (IL-1a), IL-b, C-X-C motif chemokine ligand 2 (CXCL2*) and *colony-stimulating factor 3 (CSF3)*] and one gene (*SLC1A4*), an amino acid transporter, was modulated (fold change 2, P< 0.05) in the TRF-treated cells. With a more stringent analysis (fold change 3, P < 0.05), only one gene (*CSF3*) was downregulated in the TRF-treated in RAW 264.7 cells. Analysis using the GO and KEGG pathways revealed interactions between pro-inflammatory agents such as tumor necrosis factor-alpha (TNFα) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-□B), as well as signaling pathways of interleukin (IL)-1 and IL-17.
Conclusion: TRF modulated the expression of genes responsible for acute and chronic inflammation that were part of the lipoxygenase (LOX) and cyclooxygenase (COX) inflammatory pathways. Further investigation on the effects of TRF in different cell lines and *in vivo* studies should be conducted in the future.

Keywords: Tocotrienol-rich fraction (TRF); inflammatory response; macrophages; gene regulation; ontology; KEGG pathway.

1. INTRODUCTION

Inflammation is an immunological response of our body to defend itself against injury, infection or allergy due to invasion by foreign pathogens or toxic compounds [1]. Usually, the signs of inflammation are recognized through redness, warmth, pain, swelling, tumor and loss of function [2]. Body inflammatory defense is triggered by the release of pro-inflammatory cytokines and chemokines, activated by macrophages and neutrophils that possess a crucial role in phagocytosis [2], which aims to heal the affected tissues. If the reaction is over-expressed or persists longer than necessary, this could promote inflammation-related diseases, such as ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease (NAFLD), autoimmune and neurodegenerative conditions [3].

Lipopolysaccharide (LPS) is an endotoxin, which is composed of lipid-A, O-antigen and hydrophilic core polysaccharides [4]. It is a component in the outer membrane of Gram-negative bacteria [5] and is more stable than the classical bacterial exotoxin in inducing specific symptoms and pathologies of diseases [4]. LPS is widely used in systemic inflammation in vitro and in vivo research models [4] as it can induce inflammation and result in the production of inflammatory cytokines, such as interleukin(IL)-6, IL-8, IL-1β and tumor necrosis factor-alpha (TNF-α) in various cells [5, 6].

Macrophages provide first line of host defense against microbial infections. Upon stimulation by LPS, macrophages secrete cytokines and chemokines that activate the inflammatory response to bacteria by recruiting phagocytes to the inflammatory sites [7].Additionally, LPS induces expression of tissue factors on macrophages; which are crucial for activation of the clotting cascade to limit spread of bacterial infection [7]. Macrophages also produce inflammatory mediators in response to LPS, such as nitric oxide and arachidonic acid metabolites [7]. Excessive release of these inflammatory mediators can result in septic shock, multiple organ failure, and acute respiratory distress syndrome [8].

Tocotrienol (T3), a major component of vitamin E from palm oil, has unique antioxidant and anti-inflammatory properties [9], which are beneficial in inflammation-associated diseases [10]. Several studies have shown that T3 acts as a potent inducer of apoptosis in many cancer cells [11,12,13]; possess immune regulatory roles, which reduces the risk of respiratory infections and allergy [14,15]; confer protection against oxidative stress [16,17]as well as cardiovascular [15] and neurodegenerative [17] diseases. Therefore, this study aimed to evaluate how T3 cause anti-inflammatory responses using a cell-based inflammation model, i.e. LPS-stimulated RAW 264.7 macrophage cells.

A previous study [5, 18] showed that cyclooxygenase-2(COX-2) expression was downregulated by all T3 analogues, with the best inhibition observed with delta-T3 (δT3) [18]. The same study also reported that δT3 was the most

effective anti-inflammatory agent amongst the different forms of vitamin E tested and it suppressed production of Interleukin-6 (IL-6), nitrite oxide (NO) and prostaglandin E2 (PGE2) in the LPS-stimulated RAW 264.7 macrophages [19].LPS-stimulated RAW 264.7 macrophages demonstrated that delta-tocotrienol is the most effective anti-inflammatory agent among all vitamin E forms tested and able to suppress the production of Interleukin-6 (IL-6)nitrite oxide (NO) and prostaglandin E2 (PGE2)[18].

The aim of this study was to understand how TRF induces anti-inflammatory effects by identifying the major genes that were modulated in LPS-stimulated RAW 264.7 cells treated with TRF. The microarray analysis will facilitate the elucidation of mechanisms of the molecular targets related to inflammation modulated by tocotrienols.

2. MATERIALS AND METHODS

2.1 Cell Preparation

Macrophages RAW 264.7 was purchased from the American Type Culture Collection (ATCC). The RAW 264.7 cells were grown in a DMEM medium with 5% FBS, 1% glutamine, and 1%penicillin and Streptomycin. Confluent cells were plated down in 24-well plates in a low serum medium and incubated overnight at 37°C and 5% of CO_2 to allow the cells to attach to the flask surface. The RAW 264.7 were treated with LPS (10 ng/mL) in the presence of absence of TRF (10 µg/mL) or alpha-tocopherol (α-T) (10 µg/mL) for 24 hours. There were four study groups: (i) control cells (without LPS stimulation); (ii) LPS-treated cells; (iii) TRF-treated LPS-stimulated cells and (iv) α-T treated LPS-stimulated cells. Cells without LPS stimulation served as the negative control, while the cells exposed to LPS (10ng/mL) only served as the positive control.

2.2 Ribonucleic Acid (RNA) Extraction

The total RNA was extracted from the treated and control RAW264.7 cells using the Tri-reagent (supplier, country). Briefly, 1 mL of the Tri-reagent was added to the T75 flask that contained the test, and the flask was incubated for 1 min to allow detachment from the flask. The cells were then mixed and incubated for 5 min. About 200 µL of chloroform was added into 1 mL of cells and shaken vigorously for a minute. After that, the cells were incubated at RT, put into 1 mL of cells and shaken vigorously for a minute. The cells were incubated at RT for 5 min and centrifuged at 9000 rpm at 4°C for 30 min. The RNA that remained in the aqueous phase were collected into tubes, and 500 µL of isopropanol (IPA) was added to the cell suspension. The cells were mixed and incubated for 1 min at RT before centrifugation (9000 rpm at 4°C for 15 min). The pellet was rinsed with 75% ETOH and recovered by centrifugation and suspended in double-distilled water (ddH_20) before it was stored at -20°C.

2.3 Quantity and Integrity RNA

The RNA concentration of each sample was determined using UV measurement (NanoDrop 1000, Peqlab Erlangen, Germany). The integrity of the RNA was checked using a bioanalyser (Experion system, Bio-Rad, USA) using the RNA StdSens chip (Bio-Rad, USA).

2.4 Microarray Experiment

The RNA samples(n=3 for each sample) from the four study groups [(i) negative control; (ii) positive control; (iii) TRF-treated LPS-stimulated cells, and (iv)α-T treated LPS-stimulated cells] were analyzed using the microarray approach. Briefly, the RNA (500 ng) from each sample was amplified using the Ambion Total Prep RNA amplification kit (Ambion, USA). The microarray analysis was carried out at the Malaysian Genome Institute (MGI) using an 8-bead chip platform array from Illumina (Mouse-Ref-8-V1 P/N: 11239615) in triplicate (n=3 for each sample). For the analysis, Bead Studio 2.0 from Illumina was used for the dendrogram pathway and control summary. Gene Spring 7.1 was used for the complete analysis to determine the gene expression data/results.

2.5 Quantitative RT-PCR

DNAse-treated RNA samples (n=3 for each sample) were used for the quantitative PCR (qPCR) analysis to quantify the expression of the *Hamp, IL-1a, IL-b, C-X-C motif chemokine ligand-2* (*CXCL2*) and *colony-stimulating factor 3* (*CSF3*) genes. The qPCR reaction mix contained 25 µL qPCR reaction mix (Invitrogen), 5µL of cDNA (20 ng), 0.5 µL of both sense and anti-sense primers (100µM), 0.5µL of enzyme mix, 15µL of 2x reaction (including SYBR Green dye) and 4.25µL of PCR-grade water. The denaturation step was 2 min at 94°C, and the qPCR assay was carried out for 40 cycles with

denaturation of 1 min at 94°C, annealing for 30 sec at 60°C with an extension of 30 sec at 72°C. The specified mode of reaction was controlled using the melting curve. The qPCR analyses were performed using a thermocycler (iCycler iQ5 real-time RT-PCR detection system, Bio-Rad, USA). The ΔC_T-Values were calculated relative to the house-keeping gene [*beta-actin* (*β-actin*)], and the ΔΔCT values were calculated by comparison of LPS-stimulated to treatment samples.

2.6 Enrichment Analysis using STRING Database

Further analysis of the differentially expressed genes was conducted using STRING [an online functional protein association network *software (https://string-db.org)*]. The differentially expressed genes were also analyzed using the Gene Ontology and KEGG pathways to study the pathway-related diseases that compute a functional enrichment analysis in the background and protein network.

2.7 Statistical Analysis

The statistical analysis was done using SPSS version 20. The RT-PCR data was analyzed using One-Way ANOVA, Post Hoc test with ±SD of triplicate measurement. The Bead Studio data analysis software, GENE Spring 7.0 and STRING v11 database, were used for microarray analysis.

3. RESULTS

3.1 Dendrogram Tree

The dendrogram analysis performed using the Bead Studio 2.0 software (Illumina, USA) showed that the differentially expressed genes from the TRF+LPS, LPS and α-T+LPS groups were similar as these were grouped under the same gene tree (Figure 1). In contrast, the result from the control group was located under a different branch, suggesting that the genes expressed by these cells were different (Fig. 1).

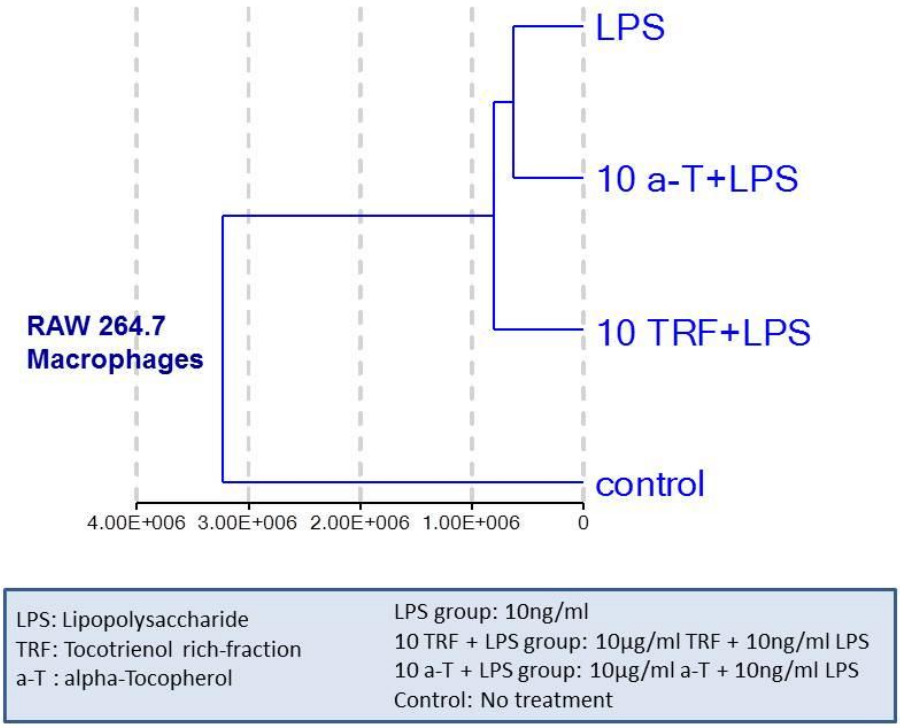


Fig. 1. Dendrogram analysis of the differentially expressed genes from the four study groups

3.2 Condition Tree

The condition tree showed the expression of genes in each group. The samples were analyzed using a third-party software program [Bead Studio 2.0.]. Figure 2 shows that the genes in the control group indicated the polled of a gene expressed in all treatments. TRF group and control group generally expressed the same gene expression because they fall in the same branch group. The genes showed that the TRF and control groups generally expressed the same gene expression because they fall in the same branch. Whereas, LPS group mostly expressed the same gene expression as in the a-T group.

3.3 Differently Expressed Genes

Six genes were differentially expressed at two-fold (P<0.05) change in the TRF vs LPs treatment groups (Fig. 3A); where one gene (*SLC1A4*) was upregulated and five genes (*Hamp, IL-1a, IL-b CXCL2*and *CSF3*) were downregulated. Only one gene (Csf3) was downregulated with three-fold change (P<0.05) (Figu. 3B). The gene list, descriptions and fold changes of each of these six genes are shown in Table 1.

3.4 Validation of Gene Expression

The expression of six differentially regulated genes (*SLC1A4, Hamp, IL-1a, IL-b, CXCL2* and *CSF3*) in the RAW 264.7 cells following exposure to LPS and/or vitamin E was verified using qPCR analysis. The validation of genes involve the treatments between LPS and TRF groups, since it has been found regulated up and down through microarray analysis. The expression trend of all six genes was consistent with the RNA-Seq data, where five genes were downregulated (*Hamp, IL-1a, IL-b, CXCL2* and *CSF3*), and one gene was upregulated (*Scla4*) (Fig. 4).

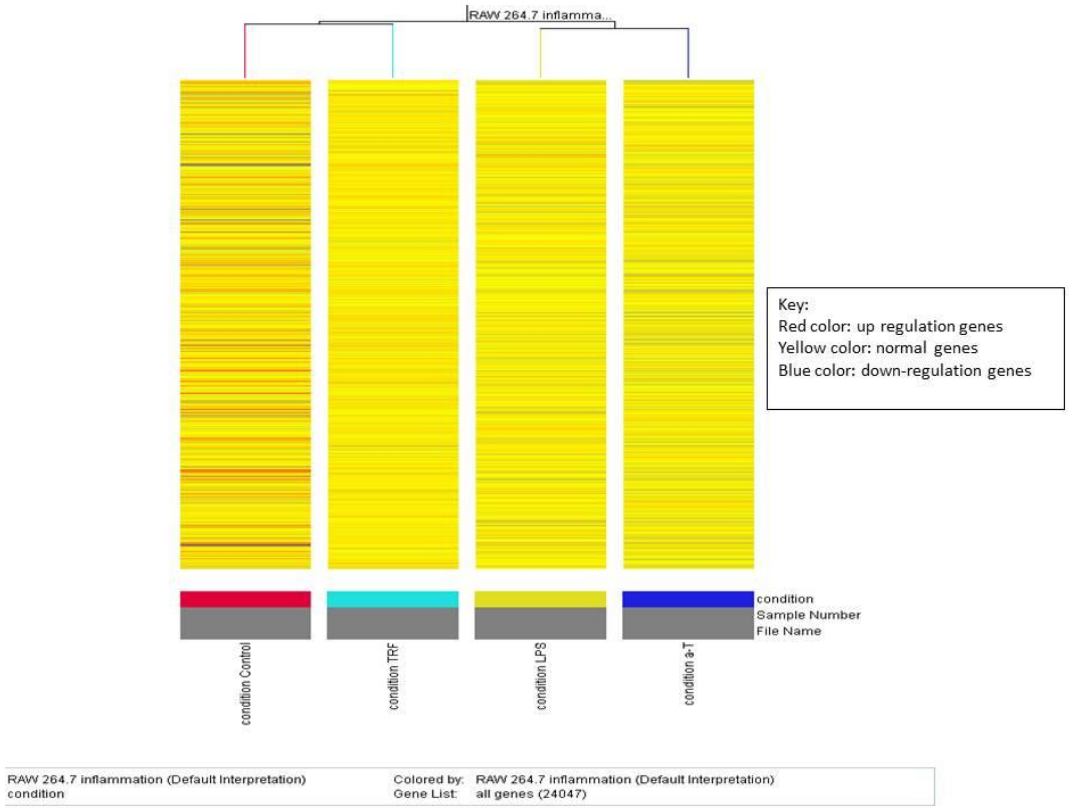
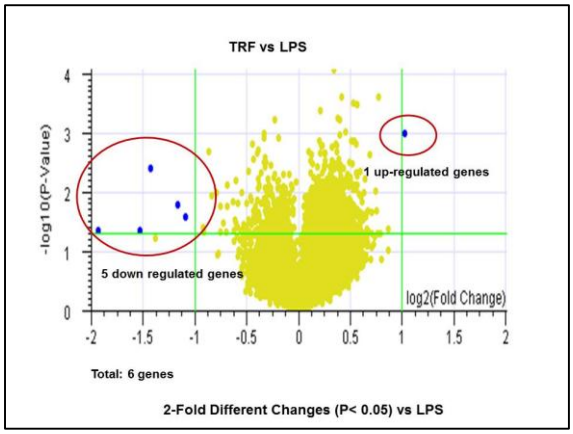


Fig. 2. Condition tree showing the condition of genes expressed in (i) condition Control (negative control-untreated cells); condition TRF (TRF-treated LPS-stimulated cells); (iii) condition LPS (positive control-LPS-treated cells); (iv) condition α-T (α-T treated LPS-stimulated cells). The condition tree was created using the Bead Studio 2.0 software program

(A) two-fold (P<0.05)



(B) three-fold (P<0.05)

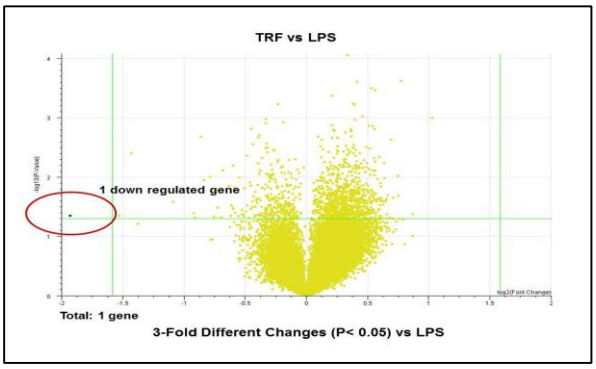


Fig. 3. Volcano plot showing (A) two-fold changes and (B) three-fold in upregulated and downregulated genes (P<0.05 = 95% significant difference)

Table 1. Genes that were significantly regulated in the Volcano plot showing (A) two-fold or (B) three-fold changes (P<0.05)

(A) two-fold (P<0.05)

No	Gene Name	Description	Regulation in TRF
1.	Slc1a4 solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Mus musculus solute carrier family 1 (glutamate/neutral amino acid transporter) member 4 (Slc1a4) mRNA.	Up-regulated
2.	Hamp hepcidin antimicrobial peptide 1	Mus musculus hepcidin antimicrobial peptide (Hamp) mRNA.	Down-regulated
3.	Il1b interleukin 1, beta	Mus musculus interleukin 1 beta (Il1b) mRNA.	Down-regulated
4.	Il1a interleukin 1 alpha	Mus musculus interleukin 1 alpha (Il1a) mRNA.	Down-regulated
5.	Cxcl2 chemokine (C-X-C motif) ligand 2	Mus musculus chemokine (C-X-C motif) ligand 2 (Cxcl2) mRNA.	Down-regulated
6.	Csf3 colony stimulating factor 3	Mus musculus colony stimulating factor 3 (granulocyte) (Csf3) mRNA.	Down-regulated

(B) three-fold (P<0.05)

No	Gene Name	Description	Regulation in TRF
1.	Csf3 colony stimulating factor 3	Mus musculus colony stimulating factor 3 (granulocyte) (Csf3) mRNA.	Down-regulated

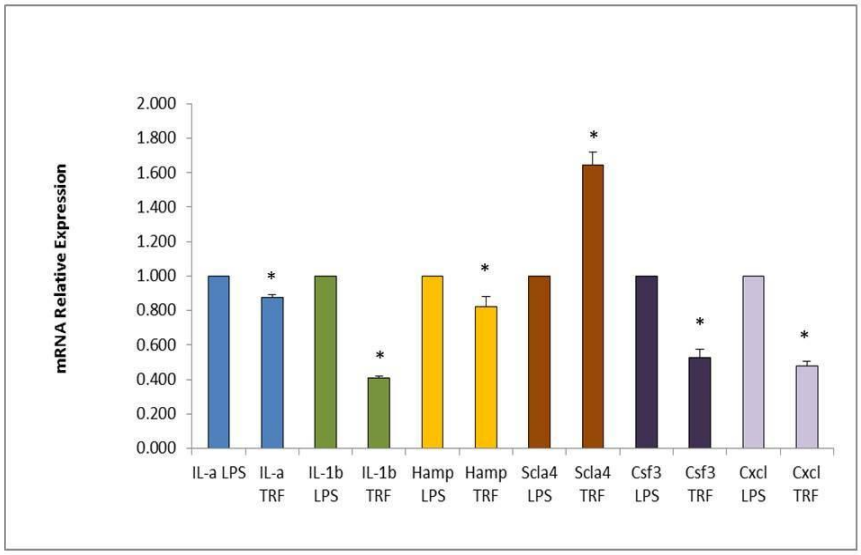
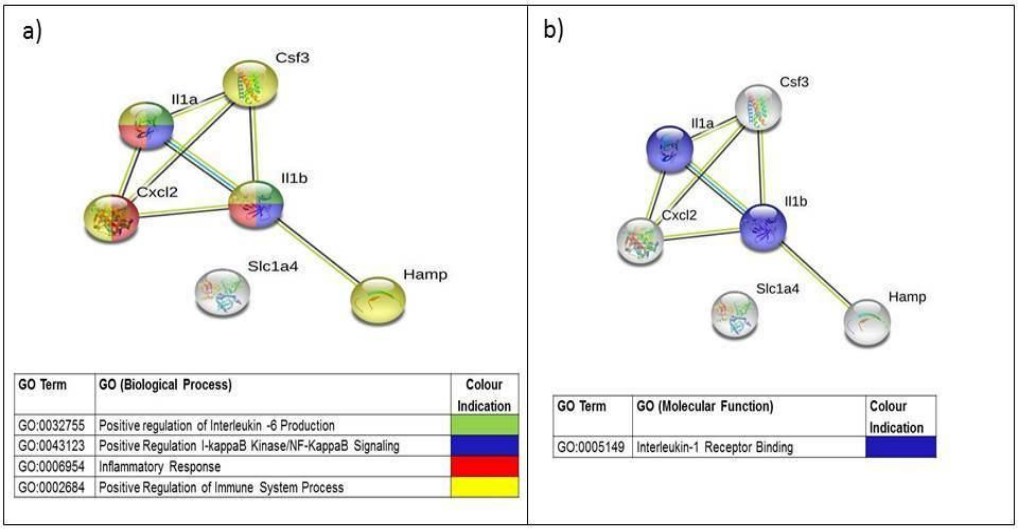


Fig. 4. Expression for the six differentially regulated genes identified from microarray analysis using quantitative PCR. The expression of genes is in relative to the expression observed to the untreated cells (LPS)

3.5 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways

According to the GO pathway analysis, under the GO (Biological Process) pathway, there were four GO terms, i.e. positive regulation on IL-6 production (GO: 0032755); positive regulation I-Kappa-B kinase/NF-κB signaling (GO: 0043123); inflammatory response (GO: 0006954) and positive regulation of immune system Process (GO: 0002684) (Fig. 5a). Furthermore, similar genes were expressed in GO: 0032755 and GO: 0043123, representing two genes (*IL-1a* and *IL-1b*), that were inter-related. For the inflammatory response (GO: 0006954), three genes (*IL-1a, IL-1b* and *CXCL2*) were involved, and there were five genes involved in the GO pathway under the positive regulation of the immune system process. Fig. 6b shows the GO under the molecular function. Only two genes were found to be inter-related for IL-1 receptor (IL-1R) binding (*IL-1a* and *IL-1b*). The GO analysis suggests that most of the gene pathways regulated by TRF in the LPS-stimulated RAW 264.7 cells were from the IL-6 and NF-□B signaling pathways.

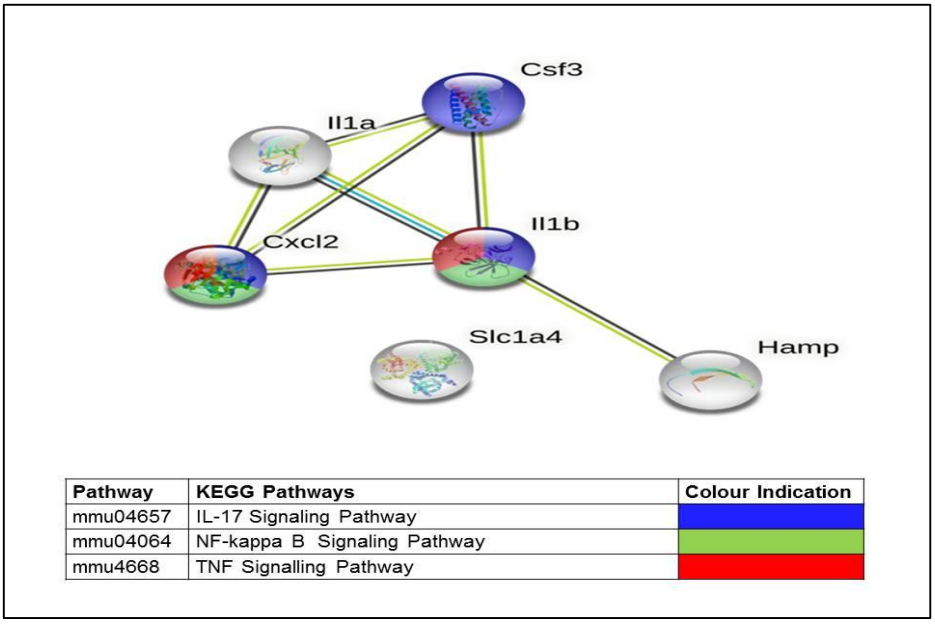
The KEGG pathway mapping was utilized based on the KEGG ontology (KO) terms. The KO assignments were used to classify the functional annotations of the identified genes to understand the biological function (Fig. 5). There were three significantly enriched KEGG pathways identified in the TRF versus LPS group, mostly linked to inflammation, such as the IL-17 signaling pathway, TNF signaling pathway and NF-□B pathway. These three enriched functional pathways were related to inflammatory responses, and the interaction was co-related with CSF3, IL-1b and CXCL2. The findings suggest that TRF may inhibit inflammatory pathways by modulating the expression of CSF3, IL-1b and CXCL2 genes via the IL-17 signaling pathway. In addition, CXCL2 and IL-1b are closely related through NF-□B and TNF signaling pathways.



GO Term	GO (Biological Process)	Colour Indication
GO:0032755	Positive regulation of Interleukin -6 Production	
GO:0043123	Positive Regulation I-kappaB Kinase/NF-KappaB Signaling	
GO:0006954	Inflammatory Response	
GO:0002684	Positive Regulation of Immune System Process	

GO Term	GO (Molecular Function)	Colour Indication
GO:0005149	Interleukin-1 Receptor Binding	

Fig. 5. Gene Ontology (GO) related to six genes from the microarray analysis associated with inflammation and immune responses



Pathway	KEGG Pathways	Colour Indication
mmu04657	IL-17 Signaling Pathway	
mmu04064	NF-kappa B Signaling Pathway	
mmu4668	TNF Signalling Pathway	

Fig. 6. KEGG pathway from six genes identified from the microarray analysis related to the inflammation pathway

4. DISCUSSION

Analysis of the microarray data showed that six differentially regulated genes in LPS-stimulated RAW 264.7 cells treated with TRF (Figure 3, Table 1), where the one gene (*SLCLA4*) was upregulated and five genes (*Hamp, IL-la, IL-1b CXCL2* and *CSF3*) were downregulated when compared to LPS-alone group. These genes were mainly involved in mediating inflammatory processes [19]. The STRING online tool analysis showed the genes' enrichment analysis with GO and the KEGG pathway (Figure 5and 6). The GO analysis is the representation of gene or gene product attributes across all species.

The *SLC1A4* gene codes for a Na-dependent neutral amino acid transporter, also known as the amino acid exchanger ASCT1, is a major d-serine uptake system in astrocytes and can also

export l-serine via hetero-exchange, supplying neurons with the substrate for d-serine synthesis [19]. Mutations in the SLC1A4 gene is associated with neurodevelopmental alterations, including lower hippocampal and striatal volumes and changes in the expression of neurodevelopmental-relevant genes [19,20] and can cause novel neurologic disorders with significant intellectual disability, severe postnatal microcephaly, spasticity and thin corpus callosum [19]. Treatment with TRF caused upregulation of the SLC1A4 gene in the LPS-stimulated RAW 264.7 cells, confirmed by qPCR analysis. The finding suggests that TRF treatment increased the expression of amino acid transporter and transfer across the plasma membrane in the RAW 264.7 macrophages, which is responsible for the traffic and balance of amino acids within and between cells and tissues, intracellular energy metabolism, anabolic and catabolic reactions in the body system [21].

Regulation of iron metabolism can be interrupted during infection and inflammatory diseases. For instance, hepcidin is a regulator for entry of iron into the body circulation. During inflammation, hepcidin level increased due to iron-trapping within macrophages and liver cells; causing decreased absorption of iron by the gut [22]. Inflammatory mediators such as IL-6 and bacteria-derived LPS trigger hepcidin production, which causes retention of intracellular iron; thus, preventing iron absorption and this can lead to ion-deficiency [23]. In this study, we found that TRF inhibited the expression of pro-inflammatory gene, such as *Hamp* (Figure 3 and 5).

Other inflammatory genes such as IL-1a, IL-1b, members of the IL-1 cytokine family and important inflammatory response mediators, are involved in various cellular activities, including cell proliferation, differentiation and apoptosis [24]. Activation of cyclooxygenase-2 (COX2) by these pro-inflammatory cytokines in the central nervous system (CNS) can cause inflammatory pain hypersensitivity [25,26]. Some studies reported that patients with severe coronavirus-19 (COVID-19) infection had higher levels of pro-inflammatory cytokines in their bronchial alveolar lavage fluid samples [27,28]. It appears that the inflammatory response in the damaged lungs induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was due to high levels of IL-1b [27,28]. In the present study, we showed that exposure to TRF downregulated the expression of IL-1a and IL-1b genes in RAW 264.7 cells (Fig. 3).

The macrophage inflammatory protein-2 (MIP-2), also known as the CXCL2, is a critical chemokine for neutrophils that is produced in the rat intestine in response to platelet-activating factor (PAF) and to mediate intestinal inflammation and injury [29].CSF3 is a co-stimulator of inflammation and autoimmunity and is associated with changes to the immune environment in colorectal cancer animal models [30]. This study showed that *CXCL2* and *CSF3*genes were downregulated in the TRF-treated group compared to the LPS-only group. Inhibition of these genes reduced inflammation in several diseases [30].

From the GO pathway classified into biological processes (Figure 5a), exposure to TRF modulated the expression of five genes (*Hamp, CXCL2, CSF3, IL-1a* and *IL-1b*) that were involved in the regulation of the immune system. Three of these genes, *CXCL2, IL-1a* and *IL-1b*, were clustered together into the inflammatory responses category in the GO-biological process; while two genes were involved found to be involved in the NF-□B signaling pathway, which is responsible for IL-6 production. On the molecular function for GO (Figure 5b), two genes were found to be co-related to each other with respect to the IL-1 co-receptor binding. IL-1 is a key signaling molecule in both innate and adaptive arms of the immune systems; mediating inflammatory responses to a wide range of stimuli [31]. This cytokine-receptor complex recruits a secondary receptor, which leads initiates several processes that results in the activation of the NF-□B pathway [31]. So, understanding the IL-1 family cytokine signaling, can benefit the treatment of some chronic inflammatory diseases due to dysregulated IL-1 signaling.

The KEGG pathway map identifies biological pathways enriched in a gene's list and are developed to focus on the changes of expression in groups [32]. The KEGG pathway analysis revealed that differentially expressed genes from microarray analysis in RAW 264.7 macrophages treated with TRF were highly associated with IL-17, NF-□B and TNF signaling pathways (Figure 6). Three genes, namely *CSF3, IL-1b* and *CXCL2*, were reported to interact strongly with the IL-17 signaling pathway. IL-17 is the founding member of a novel family of inflammatory cytokines [33]. However, the pro-inflammatory properties of IL-17 are critical to its host-protective capacity, whereby unrestrained IL-17 signaling is associated with immunopathology,

autoimmune disease and cancer progression [34]. IL-17A promotes inflammation by inducing various pro-inflammatory cytokines and chemokines, recruiting neutrophils, enhancing antibody production and activating T cells. The expression of IL-17A is augmented in autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis [35]. Furthermore, the IL-17 signaling pathway initiates signaling through Act1-induced K63-linked ubiquitylation of TRAF6, thereby activating the mitogen-activated protein kinase (MAPK), CCAAT enhancer-binding protein β (C/EBPβ) and NF-□B pathways [36]. The IL-17 family, a subset of cytokines consisting of IL-17A-F, plays crucial roles in both acute and chronic inflammatory responses. Additionally, the IL-17 family signals via their correspondent receptors and activates downstream pathways that include NF-□B, MAPKs and C/EBPβs to induce the expression of antimicrobial peptides, cytokines and chemokines [36].

The NF-□B and TNF signaling pathways were regulated in CXCL2 and IL-1b genes (Figure 6). TNF and NF-□B are cytokines belonging to the TNF family, which induce rapid transcription of genes regulating inflammation, cell survival, proliferation and differentiation, primarily through activation of the NF-□B pathway. Both belong to the COX2 pathway in the inflammatory process [37]. From the analysis of molecular interaction and networking, it is suggested that most of the pathways lead to inhibition of COX2 in the inflammation process because the NF-□B and TNF are products of the COX2 pathway, whereas IL-17 is a dual program that is involved in LOX and COX pathways [38,39].

5. CONCLUSION

In conclusion, this study showed that TRF possesses anti-inflammatory properties and downregulated genes responsible for acute and chronic inflammations. The results of this study suggested that TRF inhibited both LOX and COX in the inflammatory pathways proposing that through supplementation of TRF in RAW 264.7, macrophages would inhibit inflammatory genes via biological processes, interactions of TNF and NF-□B, as well as through inflammatory mediators such as IL-1 and IL-17. Further investigation on the effects of TRF in different cell lines and in-vivo studies should be conducted in the future.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

FUNDING

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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