Dry and Wet Lab for Analyzing an Inflammatory Responsive Gene in Lipopolysaccharide Stimulated Macrophage Cell Line

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Abstract

Current life science research often requires data analysis in a dry lab before the validation of their results in a wet lab, especially the study of microarray datasets. In a study of gene expression data for tens of thousands of genes simultaneously under favorable conditions using microarray technology, the process of selecting responsive genes is important. In this study, inflammatory responsive genes were investigated by analyzing public microarray gene expression data of LPS-stimulated RAW 264.7 macrophage cells. Three microarray datasets with four-time points (3, 6, 8, and 18 hours) were retrieved from a public database. Microarray data analysis with dry lab process includes feature selection, co-expression network analysis, gene expression profile cluster analysis, and protein-protein interaction network analysis were used in this study. The selected inflammatory responsive gene, *SOCS3*, was validated in the wet lab with real-time RT-PCR. The *SOCS3* gene showed a very volatile change in expression, which was different from the microarray data that showed an increase as time changed. This finding may be due to the induction of the *SOCS3* gene that requires co-stimulation of the stimulus to enhance expression stability. The SOCS3 protein is an important protein that regulates the negative response of cytokine signaling that helps prevent immune system disorders and inflammatory disease. Because SOCS3 protein is active at the protein level, protein expression determination may be more reliable and unravel uncertainties suggested in the analyses of the wet lab with only real-time RT-PCR.

Keywords: *Dry lab, Wet lab, Inflammation, Microarray, Real-time RT-PCR, SOCS3*

1. Introduction

Genes, in particular target genes with the potential to change their expression, hold the keys to understanding the molecular basis of inflammation. A large quantity of gene expression information is contained in microarray data. Microarray is a high throughput technology with a hybridization-based method for studying global transcriptional profiling and is widely used to understand the genetic regulation of a particular cell type (Rani & Sharma, 2017). Public microarray data can be retrieved for the identification of differentially expressed genes (DEGs) at different states of interest (Yu et al., 2020). These microarray datasets are available in public databases such as Gene Expression Omnibus (GEO) (Edgar et al., 2002; Barrett et al., 2013), which are related to the macrophage RAW 264.7 response induced by lipopolysaccharide (LPS) (Comer et al., 2006; Hammer et al., 2010; Lee et al., 2013). Finding target genes with changed expression associated with inflammation is important to understanding the response mechanism. Further, in microarray data analysis to isolate and compare genes that may be responsible for a disease error is minimized by selecting only genes important to the conditions of interest (Selvaraj & Natarajan, 2011). Currently, gene selection is achieved by statistical methods that have been challenged in terms of important gene loss. This includes integrative analysis with several bioinformatics databases and tools to select important genes of macrophage RAW 264.7 that respond to LPS over time for validation (Cline et al., 2007; Huang et al., 2009; Kanehisa et al., 2010; Selvaraj & Natarajan, 2011). Integrated analysis of multiple microarray studies identified novel gene signatures that contribute to the understanding of comprehensive molecular changes in complex disorders (Jia & Zhai, 2019). There are several publicly available software tools and web

applications for analyzing cellular pathway activity, identifying sub-network perturbations, key regulatory biomolecules, predictive model building, cluster analysis and dimension reduction, and visualization of omics data (Glaab, 2018). It is anticipated this may reduce labor, time, and cost as well as in the identification of important inflammation genes and can be applied to the prevention or treatment of associated diseases.

Feature selection step is a challenge in microarray data analysis as datasets are commonly high-dimensional which means it contains several thousand genes (features) and a low number of samples and high complexity which means the direct and indirect correlation among genes (Almugren & Alshamlan, 2019). It is extremely useful to select gene sets important to the conditions under investigation (Selvaraj and Natarajan, 2011). The user-friendly based applications for selecting differentially expressed genes in high dimension datasets are developed (Glaab et al., 2009; Sangaralingam et al., 2019; Hameed et al., 2021). The most significant genes were first analyzed from the complete microarray dataset using a modular web application for microarray analysis, ArrayMining (Ducat et al., 2019). ArrayMining includes several feature selection methods to identify high differentially expressed genes (Glaab et al., 2009). Significance analysis in microarrays (SAM) (Tusher et al., 2001), Empirical Bayes t-statistic (eBayes) (Smyth, 2004), and Partial-Least-Squares based filter (PLS-CV) (Hall, 2000) are the diverse choices of methods for identifying differentially expressed genes. These methods might also suffer from incompleteness or missing some important responsive genes that have low differential expression levels (Glaab et al., 2009). The issue is being corrected by integration analysis of gene selection with the information of known inflammatory genes. It may be arriving at a comprehensive to identify low differentially expressed inflammatory responsive genes. Inflammatory responsive genes can be found with literature mining tools, i.e. PolySearch database (Cheng et al., 2008; Liu et al, 2015). Another integration analysis is to analyze with gene co-expression network (Cline et al., 2007; Yuan et al., 2017), gene expression profile cluster analysis (Nikkilä et al., 2002; Howe et al., 2011; Aydadenta & Adiwijiaya, 2018), and protein-protein interaction network analysis (Szklarczyk et al., 2015). This analysis helps to find the correlation among important inflammatory responsive genes.

Inflammation is an adaptive response to stimuli and provides for the removal of detrimental foreign matter in addition to the repair of damaged tissue (Cheng et al., 2008). Macrophages are crucial players in a variety of inflammatory responses to environmental cues since LPS is widely used in studies of inflammation and chronic inflammation. Macrophage RAW 264.7 is a model cell used to study the inflammatory response to LPS in cell walls of gram-negative bacteria (Wu et al., 2015; Han et al., 2019). TLR4 (toll-like receptor 4) signals in a cell in response to LPS stimulation (Achek et al., 2016) and can initiate two pathways such as MyD88 dependent pathway and MyD88 independent pathway (Fujihara et al., 2003). *SOCS3* gene is expressed in the STAT-induced inhibitor (SSI), also known as the suppressor of cytokine signaling (SOCS), and is a cytokine-inducible negative regulator of cytokine signaling (Yoshimura et al., 2005). The *SOCS3* gene is induced expression by LPS, IL-1, IL-6, IL-11, and IL-10 (Inagaki-Ohara et al., 2013). Also, this gene plays an important role in inhibiting TAK1 stimulation by TRAF6 and TBK1 stimulation by TRAF3 in the ubiquitination of TRAF6 and TRAF3 (Lehmann et al., 2003; Frobøse et al., 2006) and is responsible for controlling Janus kinase (JAK) activation (Yasukawa et al., 2003). This study aims to analyze the public microarray dataset for finding the inflammatory responsive genes with available bioinformatics tools in dry lab and validation the selected gene in the wet lab with real-time RT-PCR comparing to the known responsive gene in inflammation such as *iNOS*.

2. Objective

The objectives of the study are described as follows.

1) Dry lab: To select genes that play an important role in inflammatory responses when stimulated with LPS for 3-18 hr in the RAW 264.7 macrophage cell of existing microarray data set from public databases
2) Wet lab: To validate a selected gene for its association with LPS inflammatory responses using real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

3. Materials and Methods

The overview of this study is shown in Figure 1. It consists of two parts; dry and wet labs for analyzing an inflammatory responsive gene in LPS stimulated macrophage RAW264.7 cell line.

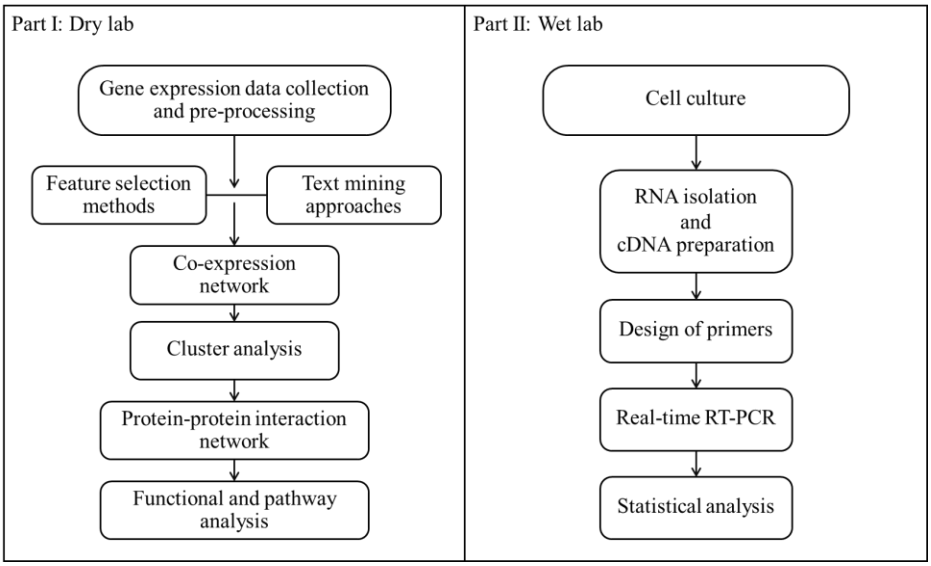

Figure 1 Overview of this study consists of two parts

Part I: Dry lab

Combination of bioinformatics databases and tools such as text mining with a gene-disease database (Cheng et al., 2008) gene co-expression network (Stuart et al., 2003) gene expression profile cluster analysis (Howe et al., 2011; Nikkilä et al., 2002), and protein-protein interaction network (Szklarczyk et al., 2015) were chosen to select important genes of macrophage RAW 264.7 that respond to LPS over time for further testing. Microarray data analyses in the selection of genes important to the inflammation process include.

3.1 Microarray Collection

Microarray data collection related to an early response time of macrophage RAW264.7 cells induced by LPS after initiation of inflammation (3, 6, 8, 18 hr). Microarray data is available from the GEO public database, Gene Expression Omnibus (Edgar et al., 2002; Barrett et al., 2013), GSE4712 (Comer et al., 2006), GSE21841 (Hammer et al., 2010), and GSE2002 (Shell et al., 2005). The dataset was selected for two groups: LPS-stimulated and non-stimulated over time-based on different platforms. Therefore, it is necessary to have a data pre-processing and normalization process to adjust values to the same standard. Z-score can be calculated from equation (1) when the mean expression value of each gene is 0, the standard deviation (SD) of the expression value for each gene set is 1 and X is an expression value for each gene in each sample within a dataset.

$$\text{Z-score}=\frac{(\text{X-mean})}{\text{SD}} \quad (1)$$

3.2 Feature selection of genes that respond to inflammation.

Gene expression data was selected for the inflammatory responsive genes stimulated with LPS over time after normalization and data preprocessing. Statistical methods are included in the online data-mining program, ArrayMining (Glaab et al., 2009). A file is uploaded into the program as specified. Each of three methods, Significance Analysis for Microarray (SAM) (Tusher et al., 2001), Empirical Bayes moderated t-statistic (eBayes) (Smyth, 2004) and Partial Least Squares Cross-Validation (PLS-CV) (Hall, 2000) were

used in genes selected by two of three methods. Data sizes are prominently displayed in the first 100 probes, each was transformed to a gene symbol for further analysis.

3.3 Selection of genes involved in inflammation.

The gene set related to inflammation was selected from the PolySearch database (Cheng et al., 2008) using text mining and parameter setting for "Inflammation" searches for 312 genes and compared to the 140 genes in the microarray source list. Inflammatory genes are analyzed together with those from the previous section to create a gene co-expression network, which was facilitated with the Expression Correlation network plugin, packaged under the Cytoscape program (Cline et al., 2007) with a cut-off value at ± 0.9 and the interesting sub-network of selected genes from PolySearch.

3.4 Protein-protein interactions and gene expression profiles

Selected genes in the sub-network were predicted for interaction between the protein and STRING database by uploading a gene or protein list that predicts the correlation and setting the interaction score parameter at 0.4 (Szklarczyk et al., 2015). Simultaneously, sub-network genes were analyzed also for gene expression profiles. Gene expression data in the sub-networks were grouped according to the expression genes contained in the MEV clustering function (Multiple Experiment Viewer) (Howe et al., 2011) SOM (Self-organizing map) was selected (Nikkilä et al., 2002). The correlation coefficient was measured by the integration of Pearsons.

3.5 Biological analysis and interpretation

Genes in sub-networks were analyzed and interpreted from a public database, DAVID (The Database for Annotation, Visualization, and Integrated Discovery: Huang et al., 2009). KEGG pathway (Kyoto Encyclopedia of Genes and Genomes: Kanehisa et al., 2010) was used to assist in the selection of inflammatory response in combination with gene expression analysis, network interaction analysis between proteins, and genetic cluster analysis at correlated expression. Selected genes were screened for validation.

Part II: Wet lab

Real-time RT-PCR is the appropriate technique for validation the selected inflammatory responsive gene, *SOCS3*. This technique includes the preparation of cDNA synthesized by using mRNA isolated from RAW 264.7 cells cultured with and without LPS. Real-time RT-PCR of three genes such as *EF-2* for the housekeeping gene, *iNOS* for the positive control gene, and *SOCS3* for the selected inflammatory responsive gene. Detail of each step in the wet lab is described below (Buapool et al., 2013).

3.6 Gene expression was validated using real-time RT-PCR.

3.6.1 Material

Dimethyl sulfoxide (DMSO), Thizolyl Blue Tetrazolium Bromide approx. (MTT) use Sigma-Aldrich (USA). D-Glucose uses Sigma (USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) use Gibco (USA). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 uses Sigma chemical (USA). Penicillin/Streptomycin uses Gibco-Invitrogen (USA). 2X iTaqTM Universal SYBR® Green Supermix uses Bio-Rad (USA).

3.6.2 Cell culture

RAW 264.7 cells, a mouse macrophage cell line, was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10000 U/mL penicillin, 10000 µg/mL streptomycin 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37 °C.

3.6.3 RNA isolation and cDNA preparation

RAW 264.7 cell, a mouse macrophage cell line, was cultured in DMEM, containing 10% FBS, and incubated in 5% CO_2 at 37 °C for 18-24 hours. Thereafter, 1 µg/mL of LPS was stimulated and re-incubated for 3, 6, 8, and 18 hours. Cells were collected and extracted for RNA using an Illustra RNAspin Mini RNA

Isolation Extract Kit. RNA concentration and purity were measured at 260 and 280 nm absorbance. Extracted RNA was synthesized for cDNA (5X iScriptTM Reverse Transcription Supermix) and subjected to quality assay by agarose gel electrophoresis.

3.6.4 Design of primers

Gene sequence information on *iNOS* (NM_010927.3), *EF-2* (NM_007907.2), and *SOCS3* (NM_007707.3) for primer design were downloaded from the National Center for Biotechnology Information. The primer size was 18-26 nucleotides in 40-60% of the PCR product length of 150-200 bp. Subsequently, sequenced primers were designed to detect gene specificity using the primer blast in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

3.6.5 Real-time RT-PCR

Synthetic cDNAs were brought to react with 2X iTaqTM Universal SYBR® Green Supermix and specific primers for each gene were mixed and reacted in real-time RT-PCR. The PCR primers were as follows.

EF-2:

5′-CTGAAGCGGCTGGCTAAGCTGA-3′ (F)
5′-GGGTCAGATTTCTTGATGGGGATG-3′ (R),

iNOS:

5′-GCACAGCACAGGAAATGTTTCAGCAC-3′ (F)
5′-AGCCAGCGTACCGGATGAGC-3′ (R),

SOC3:

5′-GCACAGCCTTTCAGTGCAGAGTA-3′ (F)
5′-GAGACAGCGGTCGTAAGAGCAG-3′ (R)

The melting curve was analyzed at 95 °C for 10 minutes and the temperature increased from 65 °C to 95 °C by adding 0.5 °C for 5 seconds.

3.6.6 Statistical analysis

All data in the control and test groups were replicated and expressed as means ± SD, using analysis of variance from several samples. Statistical significance was accepted at $p<0.05$ (Student's t-test).

4. Results and Discussion

Genes were selected in at least two of three methods with different analysis processes in the ArrayMining program (Glaab et al., 2009) with pronounced expression in the response to LPS of macrophage cells. A total of 312 genes were selected along with genes involved in the inflammation from the PolySearch database (Cheng et al., 2008). These were used to construct a co-expression network using the Expression Correlation Plugin, package under the Cytoscape program (Cline et al., 2007). Network construction found all 100 genes (nodes) and 333 interactions (edge) are shown in Figure 2A. The network was created by the data of ArrayMining containing 84 genes and 238 interactions, 11 genes with 45 interactions from PolySearch, and 5 genes with 50 interactions from ArrayMining combined with PolySearch are shown in Figure 2A. The color of the node indicates the gene source and that of the interaction line or edge indicates the interaction. The black edge indicates the same direction of the interaction and gray, the opposite direction. Subsequently, the selection of sub-network from the gene expression network was made to facilitate analysis and interpretation.

Expression genes from subnetworks selected by at least two methods including 17 genes and 50 interactions are shown in Figure 2B. The interpretation of the results to find out the function and significance of the gene's biological processes in the sub-network from the KEGG pathway database are described in

Table 1. The majorities of the genes are associated with the JAK-STAT pathway that sends signals from the outside of the cell into the nucleus, resulting in transcription and expression of immune-related genes with proliferation and apoptosis. Most cytokine receptors are expressed via the JAK-STAT pathway. JAKs are tyrosine kinases that bind to cytokine receptors and STAT is a transcription factor that combines with the cytokine receptor. This results in the dimerization of the cytokine receptors and activation of JAKs to phosphorylation to the JAKs with receptors. The STATs can bind to the receptor and phosphate is added by JAKs, resulting in dimerization of the STAT into the active form of the transcription factor that activates the expression of the gene involved in the cytokine response (Jatiani et al., 2010). Therefore, the JAK-STAT signaling pathway is important to the inflammatory response.

The selected genes involved in this pathway include four genes: *CSF2RB2*, *IL11*, *IL7R*, and *SOCS3*. The *SOCS3* gene is an interesting gene that is used to determine the level of gene expression. The *SOCS3* gene is selected from both features and the search from the PolySearch database. *SOCS3* has a high expression profile at 8 and 18 hr (Figure 2C). As a result of the predicted network interaction between proteins, the *SOCS3* gene is correlated at a protein level with JAK2 (Figure 2D), a protein that plays a key role in the JAK-STAT pathway. JAK2 is responsible for the addition of phosphate to the amino acid tyrosine to regulate cell function. *iNOS* and *EF2* gene assays to confirm inflammation have been reported to occur because the *iNOS* gene has been widely studied as being associated with inflammation (Wu et al., 2015; Buapool et al., 2013).

The mRNA analyses of the *iNOS*, *EF2*, and *SOCS3* genes were performed using real-time PCR technique to determine the cyclical threshold (C_t) for gene expression analysis. C_t is the number of cycles of the PCR reaction that can be measured by increasing the amount of DNA as shown in Figure 3. The melting curve analysis was performed to isolate different PCR products. Different types of products have different melting temperatures (T_m). The T_m of the *iNOS* gene is 82 °C, that of the *EF2* gene is 82.5 °C, and that of the *SOCS3* gene is 86.5 °C (Figure 3A). Measurement of *iNOS* and *SOCS3* gene expression by LPS stimulation at 3, 6, 8, 18, and 24 hour-intervals was used to confirm the selection of genes by microarray analysis using real-time RT-PCR. *EF-2* gene was used as a housekeeping gene.

The *iNOS* gene expression could be initiated at 3 hours post-stimulation with LPS at the highest expression level at 18 hours and until the expression at 24 hours, as shown in Figure 3C. The expression of the *iNOS* gene exhibits increased levels of expression at 3 to 24 hours. Measurement of *SOCS3* gene expression can be started with LPS stimulation at 3 hours. The expression level is highest at 8 hours but decreases to a minimum at 24 hours, see Figure 3D. The *SOCS3* gene exhibits a high degree of variability in expression, resulting in a lack of clarity over time in accord with the earlier findings of Hammer et al. (2010). The *SOCS3* gene can be induced by various stimuli such as LPS, IL-6, IL-11, interferon (IFN)-gamma, and IL-10 and may result in irregular and low levels of expressions. Induction of the *SOCS3* gene may require co-stimulation of the stimulus to enhance expression stability. For example, the induction of *SOCS3* expression by LPS with IL-10 (LPS + IL-10) is associated with a higher level of expression of the *SOCS3* gene when induced by LPS alone (Qasimi et al., 2006), which may be due to the induction of *SOCS3* expression by LPS with IFN-g (LPS + IFN-g) that has a higher level of *SOCS3* expression when induced with LPS alone (Qin et al., 2012; Qin et al., 2007).

In addition, *SOCS3* was an important response mechanism induced by LPS, IL-1, IL-6, IL-11, and IL-10 (Inagaki-Ohara et al., 2013). The mechanism of *SOCS3* gene expression in macrophage cells when stimulated with LPS in macrophage cells, the mechanism of response and signaling to pathways via Toll-like receptor 4 (TLR4), induces two pathways of cellular changes. Pathway 1: The signaling pathway required by MyD88 (MyD88 dependent pathway) is attached to TLR4 by MyD88 catches with IRAK4, IRAK1, and IRAK2, which leads to IRAK4, IRAK1, and TRAF6 activation, resulting in the auto-phosphorylation of the MyD88/IRAK4/IRAK1 complex. IRAK1 and TRAF6 are separated from the receptor complex, binding to E2 ubiquitin-conjugating enzyme complex. Ubiquitination of TRAF6 stimulates TAK1 activity and that of a transcription factor in NF-kB (consisting of IkB, p60, and p50) and AP-1 via the MAPK kinase signaling pathway (composed of ERK, JNK, and p38) and the expression of target genes such as IL-6, IL-11, IL-10, and SOCS3. TRAF6 and TAK1 are important in stimulating NF-KB and MAPK pathways by SOCS3 reduce the association between TRAF6 and TAK1. Also, SOCS3 inhibited IL-1, which was inhibited by TAK1

stimulation by TRAF6 in the ubiquitination of TRAF6 since SOCS3 can bind to Elongin B and C, components of cullin-RING E3 ubiquitin ligases (Lehmann et al., 2003; Frobøse et al., 2006; Ajibade et al., 2013). Pathway 2: TRIF dependent pathway is based on TRIF protein (TIR domain-containing adapter inducing IFN-b) by TRIF stimulates TRAF3 protein. TRAF3 will then trigger TBK1 via the ubiquitination process, which leads to the activation of the transcription factor IRFs and results in the generation of interferon-mediated inflammatory mediators (type I interferon). SOCS3 inhibits TBK1 stimulated by TRAF3 in the ubiquitination process of TRAF3 as well as in signaling pathways that require MyD88 (Kayagaki et al., 2007).

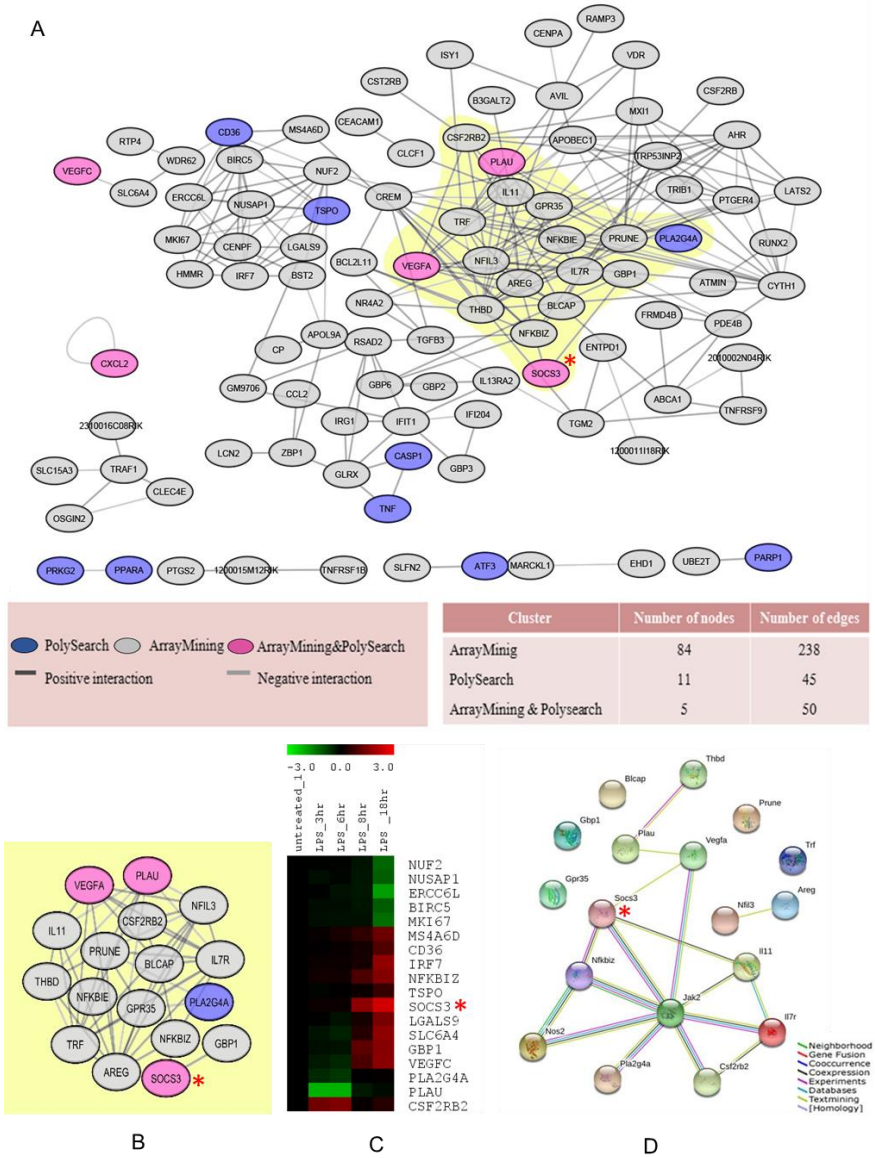

Figure 2 Dry lab: Microarray data analysis for selecting important genes in response to inflammation by creating a gene co-expression network (A), Selecting sub-network shown in the yellow shade (B), Clustering of the gene expression profile of gene in selected sub-network (C), and integrating into the database interaction between the proteins retrieved from STRING database (D). *SOCS3* gene is shown in red star.

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Table 1 Biological analysis of genes in sub-network was analyzed and interpreted from a public database, DAVID, by using the KEGG pathway.

Term	P-value	Genes
mmu04060 Cytokine-cytokine receptor interaction	4.0E-4	*CSF2RB2, IL11, VEGFA, VGFC*
mmu04630 Jak-STAT signaling pathway	6.7E-4	*CSF2RB2, IL11, IL7R, SOCS3**
mmu04640 Hematopoietic cell lineage	2.2E-2	*CD36, IL11, IL7R*

**SOCS3*, the selected responsive gene for validation in the wet lab

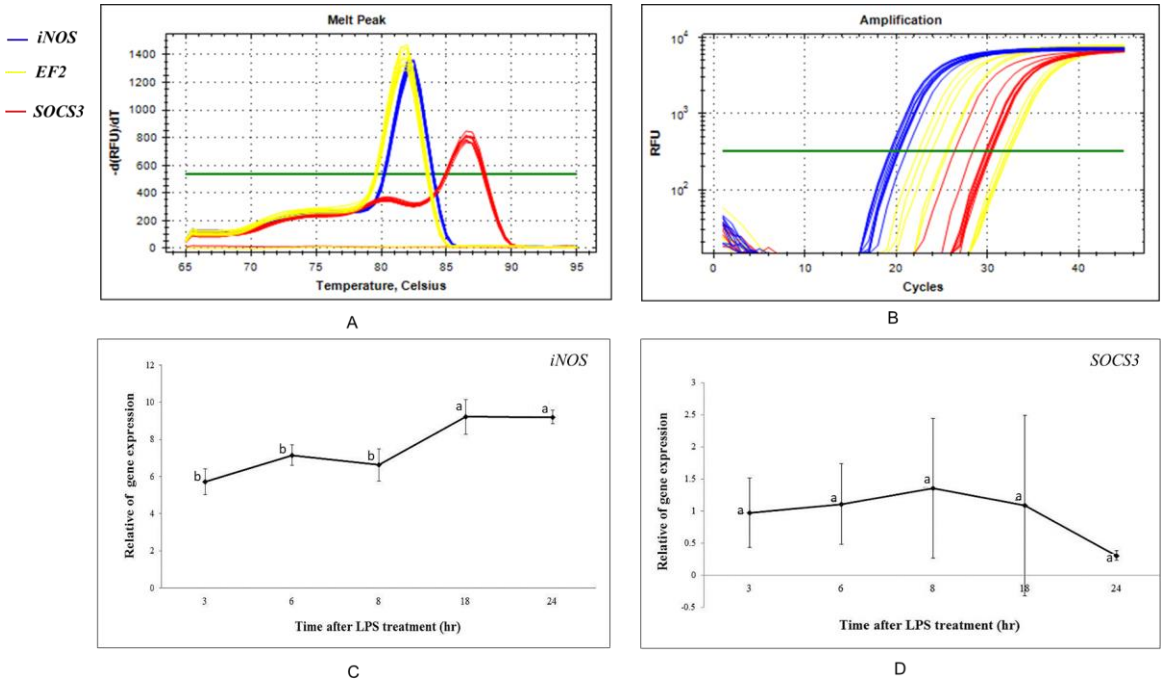

Figure 3 Wet lab: Analyzing of melting curve analysis of *iNOS*, *EF2*, and *SOCS3* genes by measuring fluorescence from PCR product at temperature increased by 0.5°C from 65°C to 95°C (A). When the scale is changed to dF/dt, it shows the melting temperature of each gene (B). The expression of iNOS (C) and SOCS3 (D) gene using real-time PCR technique in non-LPS induced macrophage RAW264.7 and LPS-induced macrophage RAW264.7 at 1 μg/ml LPS induced at 3, 6, 8, 18, and 24 hours. The data are mean ±SD of the experiment at least three times of independent each other, with $p < 0.05$ as compared to non-LPS induced cells.

IL-6, IL-11, and IL-10 induced expression of the *SOCS3* gene via JAK-STAT pathways. *IL-6* and *IL-11* genes are signaled via JAK2/STAT3 as the two genes capture the same receptor as the gp130 receptor. The *IL-10* gene is signaled via JAK1/TYK2/STAT3. All three genes signal via STAT3 stimulation, which is a transcription factor of the same type in *SOCS3* gene expression induction. IL-6 can act as an inflammatory mediator and acts as an anti-inflammatory agent. Under conditions where the *SOCS3* expression was low, IL-6 induced SOCS3 generation via JAK2/STAT3. SOCS3 regulates the action of Janus kinase (JAK) activation (Yoshimura et al., 2007). SOCS3 is bound to the JAK-proximal sites on the cytokine receptor because SOCS3 has the same receptor for IL-6 and IL-11 as the gp130 receptor that can accommodate this but not with IL-10 present. SOCS3 inhibits JAK activity via the KIR domain, which is decomposed via ubiquitination and proteasome breakdown via the SOCS box domain (Yasukawa et al., 2003; Wormald et al., 2006). In summary, the mechanism of the SOCS3 response helps prevent abnormal and inflammatory diseases of the immune system.

5. Conclusion

The microarray data were analyzed to select other genes related to the inflammation identified *SOCS3* genes as regulating the negative response of cytokine signaling that helps to prevent immune system disorders and inflammatory diseases. Genes confirmed with the real-time RT-PCR revealed that *SOCS3* exhibits considerable variability, impairing clarity in the interpretation. It may be necessary to validate the protein level and unravel uncertainties suggested in the analyses of microarray information.

6. Acknowledgements

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