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Expression Profile of Inflammatory Genes in Human Airway Epithelial A549 Cells

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Abstract

This study was conducted to evaluate the inflammation mechanisms of tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and IL-1β-induced stimulation of A549 human epithelial cells. In the present study, A549 cells were stimulated with TNF-α, IL-4 and IL-1β to induce expression of chemokines and adhesion molecules involved in eosinophil chemotaxis. The effects of TNF-α, IL-4 and IL-1β on gene expression profiles in A549 cells were evaluated by oligonucleotide microarray and Real time RT-PCR. The gene expression profiles for the A549 cells varied depending on the cytokines. Also, the results of the microarray and Real time RT-PCR revealed that inflammatory-related genes were up-regulated in cytokine stimulated A549 cells. Cytokines can affect inflammation in A549 cells. A microarray-based genomic survey is a high-throughput approach that enables evaluation of gene expression in cytokine stimulated cell lines.

Keywords: Asthma, Gene expression profile, Microarray

Respiratory epithelium is a target of the local inflammaroty response. In the initial phase of the inflammatory response, locally produced proinflammatory cytokines trigger the activation of circulating white blood cells, followed by their reversible tethering and rolling over activated endothelium, a process controlled by selectins. Later phase of this process, leukocytes migrate across the vessel wall into the underlying tissues[1,2]. In asthma and other airway inflammatory diseases, their exacerbations via the production of numerous cytokines, chemokines, inflammatory enzymes, adhesion molecules, and other mediators[3-5]. These chemokines and adhesion molecules play a pivotal role in the infiltration of inflammatory cells into the epithelium. Th2 cells also produce IL-5 which activates eosinophils in the asthmatic airway[6,7]. In addition, eosinophils express several membrane receptors. Among them are receptors for immunoglonulins, cytokines, chemokines such as eotaxin and regulated on activation in normal T cells expressed and secreted (RANTES) and adhesion receptors[8-10]. The CC chemokine subfamily is composed of 28 members. This chemokines bind to CCR1 and CCR5 and has been shown to be increased in bronchoalveolar lavage and bronchial biopsies of asthmatic patients[11]. These and many other chemokines and receptors are implicated in cell migration from the vascular compartment to the interstitium and in cell localization around airways but may also alter cell survival, proliferation, or airway remodeling[12].

The goal of this study was determine the inflammation mechanisms of TNF-α, IL-4 and IL-1β in A549 cells, a type II alveolar epithelial cell. It was hypothesized that TNF-α, IL-4 and IL-1β could induce inflammation to the A549 cells. The hypothesis of inflammatory strategy and their possible mechanisms are discussed.

Gene Expression Profiles in A549 Cells

Gene expression profiles were significantly up- or down-regulated in the ST (TNF-α, IL-4 and IL-1β stimulated A549 cells) when compared with the control (non-stimulated A549 cells). In total, 386 differentially expressed genes (213 down and 173 up) were detected in the stimulated A549 cells using approximately 54,600 oligonucleotide probes. For the stimulated A549 cells, genes showing highly altered expression levels were aligned according to the magnitude of the altered expression. Most of the differentially expressed genes are grouped into functional categories based on the KEGG database. These functional categories were responsible for processes including

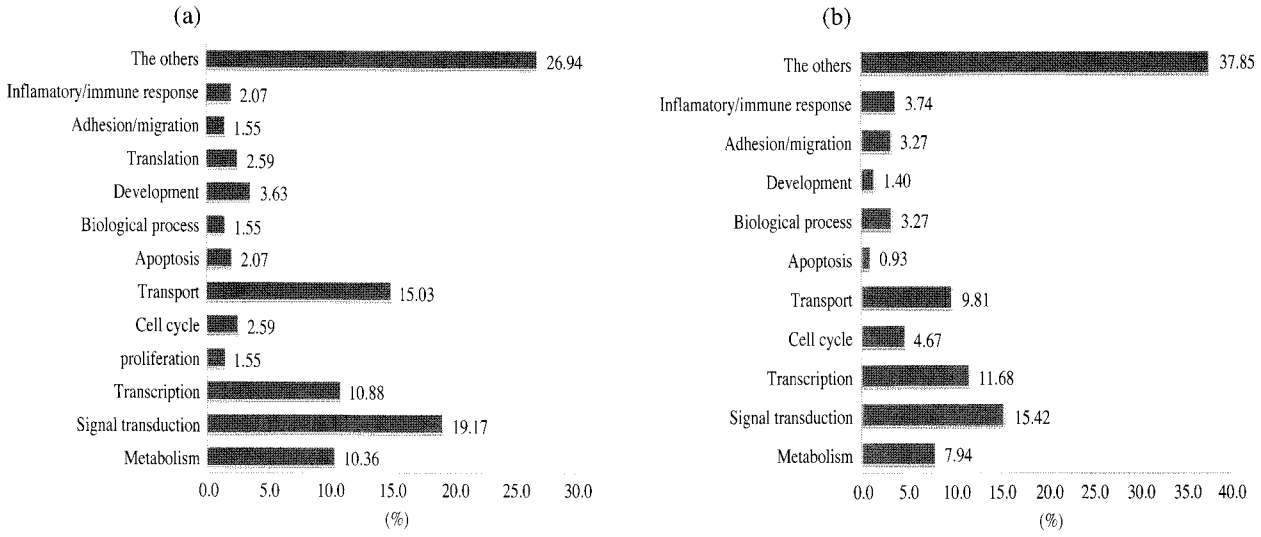

Figure 1. Gene Ontology classifications of genes based on comparison of gene expression between experimental (IL-4, IL-1β, TNF-α stimulated) and control (non-stimulated) A549 cells. (a) over-expressed genes, (b) under-repressed genes.

inflammatory and immune response, adhesion and migration, translation, development, biological process, apoptosis, transport, cell cycle, transcription, signal transduction, and metabolism, as well as other functions (Figure 1). Among these genes, inflammation pathway-related genes are listed in the Table 1, which shows a comparison of the expression levels for a variety of genes between the stimulated A549 cells and the control cells.

Real Time RT-PCR Analysis

We assessed the effects of TNF-α, IL-4 and IL-1β stimulated A549 cells for 6 h using Real time RT-PCR to measure the changes in the mRNA levels of *NOS2a, SELE, IL-1β, PDGFb*, and *FOXP3*. To normalize the expression level changes, we used the expression level of the control gene, *GAPDH*, which is known to be invariant when treated with cytokines. Real time RT-PCR revealed that the expression of *NOS2a, SELE, IL-1β*, and *PDGFb* mRNA increased significantly in stimulated A549 cells. Conversely, the expression of *FOXP3* mRNA was decreased in stimulated A549 cells for 6 h (Figure 2).

Discussion

The role of inflammation in asthma and other airway allergic diseases of the airways is widely appreciated, and airway inflammation is now included as a defining feature of asthma[13]. Our objective was to determine how cytokine effect gene expression profiles in human epitherial lung cells (A549). In this study, we used that A549 cells were stimulated with TNF-α, IL-4 and IL-1β. Pro-inflammatory cytokines such as TNF-α and IL-1β are key regulator of immune and inflammatory genes[13] and IL-4 is a pivotal cytokine, produced by Th2 cells, and associated with allergic airway diseases that support Th2 development[7]. However, the early signaling events involved in TNF-α, IL-4 and IL-1β-induced epitherial lung cells activation are not complicatedly understood, therefore we evaluated the gene expression profiles of A549 cells that were treated with TNF-α, IL-4 and IL-1β for different lengths of time (30 min, 1 h, 3 h, and 6 h). Specific and significant alterations of the expression profile of TNF-α, IL-4 and IL-1β-stimulated A549 cells were observed (Table 1).

Several CC chemokines have been shown to be up-regulated in the airway of asthmatic patients. Especially, CCR1 is activation by CCL3 or CCL23 induces the mobilization of intracellular calcium[11]. In our experiments, we detected the up-regulation of *CCL5 (RANTES), CCL8, CCL14, CCL17, CCL20, CADPS*, and *CACNA1C* in stimulated A549 cells (Table 1).

In the lungs, macrophage- and epithelial cell-derived IL-8 induces airway neutrophilic inflammation and thus could be of importance in certain forms of asthma and other airway diseases. IL-8 is a member of the CXC chemokine subfamily of cytokines and may evoke the migration of neutrophils, monocytes, and eosinophils to the sites of inflammation, injury, or infection. IL-8 mediates its effects through the activation of specific G protein-coupled receptors on target cells[14-16]. In this study, we found that *IL-8, GPR1, GPR44, GPR84, GPR98, ARHGAP15, ARHGAP24,*

Table 1. Up- and down-regulation of genes based on comparison of gene expression between experimental (ST : TNF-α, IL-4 and IL-1β stimulated) and control (NC : non-stimulated) A549 cells.

Genes	Abb.	Regulation profile and log2 ratio NC vs ST			
		30 m	1 h	3 h	6 h
interleukin 8	IL8	3.5	3.6	4.1	3.6
Immunoglobulin heavy constant alpha 1	IGHA1	3.3	2.6	3.5	3.3
programmed cell death 1	PDCD1	3.8	4.1	1.9	3.9
programmed cell death 6	PDCD6	2.9	3.3	2.1	2.5
chemokine (C-C motif) ligand 5	CCL5	1.2	3.3	8.1	8.8
chemokine (C-C motif) ligand 14	CCL14	4.3	1.0	5.2	4.3
chemokine (C-C motif) ligand 8	CCL8	3.6	2.2	5.3	5.4
G protein-coupled receptor 98	GPR98	0.5	4.5	3.6	3.0
colony stimulating factor 1 (macrophage)	CSF1	1.7	1.9	4.2	3.3
G protein-coupled receptor 44	GPR44	0.5	0.5	1.7	2.2
chemokine (C-C motif) ligand 17	CCL17	−1.0	0.8	2.9	3.4
Chemokine (C-X-C motif) ligand 2	CXCL2	6.0	6.1	5.9	6.0
chemokine (C-C motif) ligand 20	CCL20	5.6	5.6	6.2	5.5
chemokine (C-X-C motif) ligand 5	CXCL5	0.6	0.9	2.2	2.8
erythrocyte membrane protein band 4.1	EPB41	2.6	2.9	2.4	2.5
complement component (3b/4b) receptor 1	CR1	0.3	1.4	2.8	3.4
ADAM metallopeptidase domain 10	ADAM10	3.7	3.7	2.4	3.7
colony stimulating factor 2 (granulocyte-macrophage)	CSF2	6.6	7.4	9.9	7.3
Tumor necrosis factor (ligand) superfamily, member 13	TNFSF13	1.4	2.3	4.0	3.9
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	2.3	2.3	4.1	5.1
fibroblast growth factor 18	FGF18	1.6	1.7	4.8	3.3
nitric oxide synthase 2A (inducible, hepatocytes)	NOS2A	2.6	2.9	3.0	3.5
matrix metallopeptidase 15 (membrane-inserted)	MMP15	1.5	1.8	2.8	2.2
Mitogen-activated protein kinase kinase kinase 7 interacting protein 2	MAP3K7IP2	3.9	3.7	4.4	3.8
MAP/microtubule affinity-regulating kinase 1	MARK1	3.3	4.7	5.0	4.9
matrix metallopeptidase 11 (stromelysin 3)	MMP11	0.1	0.9	2.4	1.5
interleukin 2 receptor, alpha	IL2RA	2.6	2.4	3.2	3.3
interleukin 1, beta	IL1B	1.7	2.2	5.6	4.7
Rho GTPase activating protein 24	ARHGAP24	4.4	2.2	2.1	3.9
G protein-coupled receptor 1	GPR1	1.9	1.7	1.4	2.4
G protein-coupled receptor 84	GPR84	3.4	3.0	5.1	4.6
Rho GTPase activating protein 15	ARHGAP15	0.4	5.1	5.1	2.1
Rho guanine nucleotide exchange factor (GEF) 7	ARHGEF7	5.5	5.3	5.4	5.0
Ras homolog gene family, member C	RHOC	1.8	2.5	2.3	3.0
CREB/ATF bZIP transcription factor	CREBZF	1.5	1.8	1.3	2.0
MAK10 homolog, amino-acid N-acetyltransferase subunit	MAK10	3.4	2.9	1.4	1.9
ADAMTS-like 1	ADAMTSL1	0.1	1.9	2.6	1.9
Ras association (RalGDS/AF-6) domain family 5	RASSF5	−0.3	0.6	5.4	4.7
Death-associated protein	DAP	−0.1	1.6	0.6	2.0
erythrocyte membrane protein band 4.1-like 3	EPB41L3	0.1	1.7	1.7	1.9
Inositol 1,4,5-triphosphate receptor, type 2	ITPR2	1.1	2.9	3.7	4.3
phosphoinositide-3-kinase, class 2, gamma polypeptide	PIK3C2G	0.0	1.7	3.0	3.2
TYRO protein tyrosine kinase binding protein	TYROBP	3.2	3.1	2.8	2.7
erythrocyte membrane protein band 4.1	EPB41	2.6	2.9	2.4	2.5
CD209 molecule	CD209	2.0	2.0	2.4	2.1
lymphocyte antigen 75	LY75	2.9	1.2	3.1	4.0
Ca^{2+}-dependent secretion activator	CADPS	0.2	3.0	1.2	3.5
proline-rich protein BstNI subfamily 1	PRB1	1.9	1.6	1.2	1.9
Regulator of G-protein signalling 13	RGS13	0.8	2.2	1.2	4.7
SH2 domain protein 1A, Duncan's disease	SH2D1A	2.8	3.3	1.0	2.9
A kinase (PRKA) anchor protein 13	AKAP13	1.8	1.2	3.0	1.7
calcium channel, voltage-dependent, L type, alpha 1C subunit	CACNA1C	3.5	3.3	1.7	2.1
Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	2.6	1.7	2.0	3.2
phospholipase A2, group XIIB	PLA2G12B	4.4	4.4	3.8	5.0
PTK2 protein tyrosine kinase 2	PTK2	1.7	1.5	1.2	2.1

Table 1. Continued.

Genes	Abb.	Regulation profile and log2 ratio NC vs ST			
		30 m	1 h	3 h	6 h
matrix metallopeptidase 15 (membrane-inserted)	MMP15	1.5	1.8	2.8	2.2
lysyl oxidase	LOX	3.3	3.5	2.3	2.6
RAB GTPase activating protein 1-like	RABGAP1L	2.5	1.3	0.5	2.5
olfactory receptor, family 5, subfamily V, member 1	OR5V1	3.9	3.2	4.0	3.2
olfactory receptor, family 1, subfamily J, member 2	OR1J2	0.7	2.4	3.6	3.8
Son of sevenless homolog 1 (Drosophila)	SOS1	0.2	1.5	2.2	2.6
Ras homolog gene family, member A	RHOA	0.2	3.3	0.5	3.1
B-cell CLL/lymphoma 11A (zinc finger protein)	BCL11A	2.3	2.0	2.3	2.5
ADAMTS-like 1	ADAMTSL1	0.1	1.9	2.6	1.9
selectin E (endothelial adhesion molecule 1)	SELE	4.6	5.5	7.5	5.7
intercellular adhesion molecule 1	ICAM1	2.0	3.1	6.6	5.3
vascular cell adhesion molecule 1	VCAM1	1.0	1.8	3.5	3.9
platelet-derived growth factor beta polypeptide	PDGFB	1.8	1.2	1.0	1.1
forkhead box P3	FOXP3	−3.0	−1.7	−1.8	−2.6

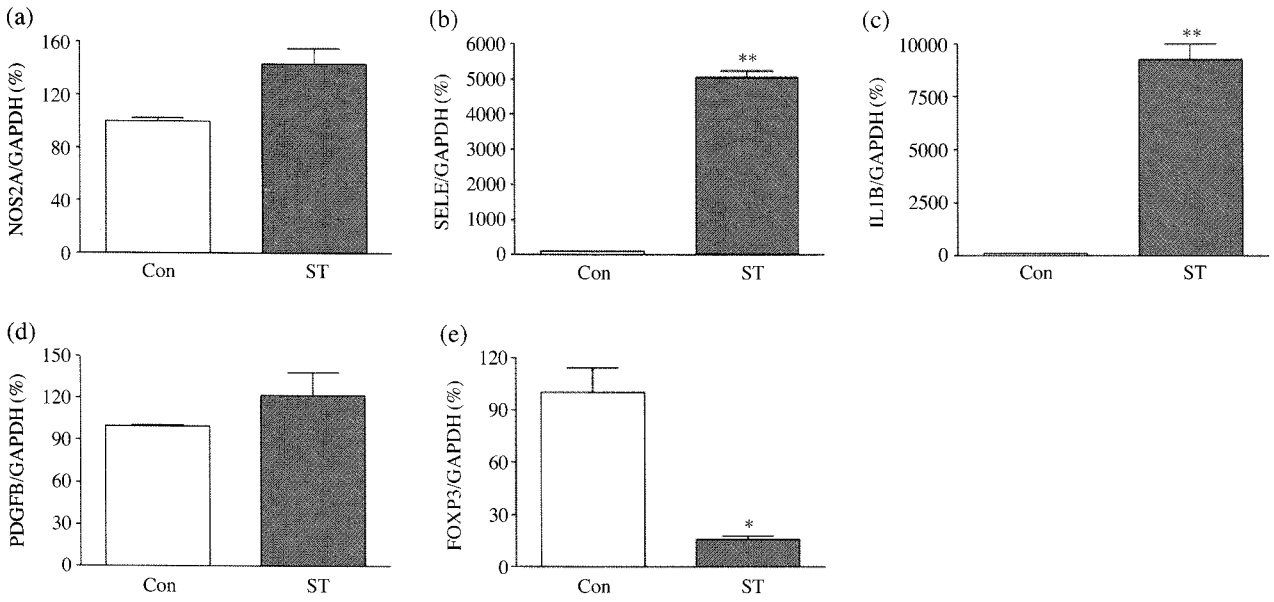

Figure 2. Effects of cytokines on mRNA levels seen by real-time RT-PCR. (a) NOS2a, (b) SELE, (c) IL1β, (d) PDGFb, and (e) FOXP3. ST : IL-4, IL-1β, TNF-α stimulated A549 cell, Con : non-stimulated A549 cell. Data are presented as mean±S.E.M. $*P<0.05$ and $**P<0.01$ compared to control.

ARHGEF7, RGS13, RABGAP1L, CXCL2, and *CXCL5* were up-regulated in stimulated A549 cells (Table 1).

The MAPK pathways are deeply involved in signaling for various immune responses such as apoptosis. MAP kinases are serine/threonine kinases, which include the extracellular signal-related kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs). Activation of the MAPK kinase pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases, which are coupled to the activation of Ras G-proteins through Src homology 2 domain-containing proteins, such as Shc and Grb2, and quinine nucleotide exchange factors such as SOS[17,18]. The results of this study revealed the up-regulation of *FGF18, PDGFb, MARK1, MAP3K7IP2, RHOA, RHOC, RASSF5*, and *SOS1* in stimulated A549 cells (Table 1, Figure 2). Each of the MAPKs has been implicated in inflammatory events and the MAPK pathways have been linked to mediation of many of the physiological responses to NO. In addition, NO regulation of matrix metalloproteinases proteins, including MMP1, during inflammatory and angiogenic

responses may require MAP kinase proteins. In our experiments, we detected the up-regulation of *MMP11*, and *MMP15* in stimulated A549 cells (Table 1). NO is a reactive free radical that generally increased during inflammatory airway disease such as asthma. This gene encodes a nitric oxide synthase which is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines[17,19-21]. In this study, we found that *NOS2a* was up-regulated in stimulated A549 cells (Table 1, Figure 2).

In conclusion, this study provides new insights in understanding the mechanisms prevailing in the establishment of inflammatory airway disease such as asthma and unveiled new potential therapeutic targets in airway allergic diseases.

Materials & Methods

Cell Culture

A549 cells (human type II-like epitherial lung cells) were obtained from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). These cells were grown and maintained in 100% humidity and 5% CO_2 at 37°C in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen Life Technologies, Rockville, USA), at 1×10^6/mL. A549 cells were then plated onto 12-well, flat-bottom tissue culture plates at a density of 5×10^5/well in hormonally defined RPMI media as described previously. The medium was changed every 2 days until the cells became 80-90% confluent, at which point they were used for experiments.

RNA Preparation

A549 cells were initially cultured in a 100 mm dish (1×10^6/mL) for 24 h, and then stimulated with TNF-α (100 ng/mL), IL-4 (100 ng/mL) and IL-1β (100 ng/mL). Next, the cells were incubated at 37°C for 30 min, 1 h, 3 h, or 6 h. RNA were then isolated from the A549 cells with Rneasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using NanoDrop (NanoDrop Technologies Inc., ND-1000; Wilmington, DE USA). The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis (data not shown).

Oligonucleotide Chip Microarray

Oligonucleotide chip microarray was performed using single round RNA amplification protocols, following Affymetrix specifications (Affymetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 micrograms of total RNA were used to synthesized first-strand complementary DNA (cDNA) using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer (Proligo LLC, Boulder, CO) and the Superscript Choice System (Life Technologies, Invitrogen, Milan, Italy). After double-stranded cDNA synthesis, products were purified by phenol-chloroform extraction, and biotinylated antisense complimentary RNA (cRNA) was generated through *in vitro* transcription using the BioArray RNA High-Yield Transcript Labeling kit (ENZO Life Sciences Inc., Farmingdale, NY). The biotinylated labeled cRNA was then fragmented, and 10 μg of total fragmented cRNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 GeneChip array (P/N900470, Affymetrix Inc., USA). The Affymetrix Fluidics Station 400 was then used to wash and stain the chips, after which the nonhybridized target was removed. Next, the samples were incubation with a streptavidin-phycoerythrin conjugate to stain the biotinylated cRNA. The staining was then amplified using goat IgG as blocking reagent and biotinylated antistreptavidin antibody (goat), followed by a second staining step using a streptavidin-phycoerythrin conjugate. The fluorescence was detected using the Genechip System Confocal Scanner (Hewlett-Packard), and analysis of each GeneChip was conducted using the GeneChip 3.1 software produced by Affymetrix, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Data Analysis

The Beadstudio was used to evaluate the expression signals generated by the Affymetrix Human Genome U133 Plus 2.0 GeneChip array. Global scaling normalization was then performed and the normalized data were log-transformed using base 2. Next, fold change was applied to select the differentially expressed genes (DEGs) using a fold change threshold of 1.5-fold and a $P < 0.05$ to indicate significance. Each probe set used in the Affymetrix GeneChip produces a detection call, with P (present call) indicating good quality, M (marginal call) indicating intermediate quality and A (absent call) indicating relatively low reliability. Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false positives. Gene ontology significance analysis was conducted to investigate the functional relationships among the 1.5-fold DEGs using high-throughput GoMiner.

Real Time RT-PCR Analysis

Microarray verification was performed by Real time RT-PCR analysis of selected genes using SYBR Green

I Master Mix (Applied Biosystems, Foster City, CA, USA) and primers (Genotech Inc., Korea). cDNA was synthesized using 2 μg of RNA in a reverse transcription reaction. Real time RT-PCR quantitative mRNA analyses were performed with an Applied Biosystems 7300 Real time PCR System using the SYBR Green fluorescence quantification system (Applied Biosystems, Foster City, CA, USA) to quantify the amplicons. The PCR conditions were 40 cycles of 95°C for 15 sec, 60°C for 1 min, and a standard denaturation curve. The sequences of the human primers were as follows: NOS2A (FW 5′-acc agg agg aga tgc tgg ag-3′; RW 5′-aac ata gag gtg gcc tgg ct-3′), SELE (FW 5′-gcc tgt gtg agc aag cat tt-3′; RW 5′-tcc tca agg cta gag cag ctt-3′), IL1B (FW 5′-cca tca gcc agg aca gtc ag-3′; RW 5′-atg tgg gag cga atg aca ga-3′), PDGFB (FW 5′-cag gca ggc tat gct gag ag-3′; RW 5′-aga ctg atg ggg tcg ctc tt-3′), FOXP3 (FW 5′-gtg act ggg atg gcc tca a-3′; RW 5′-cac cac caa caa ccc aca tc-3′), and GAPDH (FW 5′-ttc acc acc atg gag aag gc-3′; RW 5′-ggc atg gac tgt ggt cat ga-3′). PCR conditions for each target were optimized according to the primer concentration, the absence of primer dimmer formation, and the efficiency of amplification of both the target genes and the housekeeping gene control. PCR reactions were carried out in a total volume of 20 μL in PCR master mix containing 10 μL 2X SYBR Green, 5 μM each of sense and antisense primer, and 2 μL of 1 : 2 diluted cDNA filled up to 20 μL with DEPC-treated H_2O. To normalize the cDNA content of the samples, we used the comparative threshold (C_T) cycle method, which consists of the normalization of the number of target gene copies versus the endogenous reference gene, GAPDH. The C_T is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the PCR product is first detected.

Statistical Analysis

Statistical analysis of the data was conducted using the Prism 4.02 software (GraphicPad Software Inc., CA, USA). Data were analyzed by one-way ANOVA for multiple comparisons. Results with a $P<0.05$ were considered statistically significant.

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