THE EFFECT OF RAA ON mRNA DEGRADATION
IN THP-1 HUMAN LEUKEMIC MONOCYTES

by

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Abstract

Dysregulation of mRNA levels is a genetic hallmark of cancer. The Shaw-Kamen (SK) box, an AU-rich element (ARE) found in the 3'UTR, is an mRNA stability determinant in many cytokines, growth factors, transcription factors, and proto-oncogene mRNAs. A fungal metabolite, Radicicol Analog A (RAA), was found to destabilize only SK box-containing mRNAs in THP-1 human leukemic monocytes (Cytokine, 1996, 8, 751-761). Given the small number of genes analyzed in this study, SAGE was used to examine the transcriptome-wide effect of RAA on mRNA expression in THP-1 cells. To determine whether RAA only down-regulated SK box-containing mRNAs, two ~45,000-short-tag SAGE libraries were prepared from total mRNA isolated from THP-1 cells stimulated with IFNγ/LPS ± RAA (1μM). Surprisingly, only 0.27% of the total unique SAGE tags (p<0.001) were down-regulated by RAA treatment. Tag-to-gene identities for these 48 down-regulated SAGE tags were validated and quantified using real-time RT-PCR. Although 73% of these RAA-down-regulated tags encoded SK box-containing mRNAs, no linear correlation was observed between the fold decrease in mRNA expression and the number of the SK boxes. mRNA half-lives for ten genes, were determined in unstimulated, and 2 and 16 hour post-IFNγ/LPS stimulated THP-1 cells. The mRNA half-lives of two SK box-containing mRNAs, TNF-α and NFKBIA, were unaffected by RAA, suggesting that RAA may affect transcription. Interestingly, all mRNAs stabilized upon IFNγ/LPS treatment were destabilized by RAA at the post-transcriptional level. Hopefully, by studying the effect of RAA on mRNA regulation, a new approach to cancer treatment and other diseases, may be gained.
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Glossary of Abbreviations

3' UTR, 3' untranslated region
ACTB, β-actin
ActD, actinomycin D
AP-1, activator protein 1
APS, ammonium persulfate
ARE, AU-rich element
ARED, ARE-mRNA database
ATF, activating transcription factor
B2M, β-2-microglobulin
BCCA, BC Cancer Agency
βME, 2-β-mercaptoethanol
bp, base pair
BrUTP, bromouridine 5’-triphosphate
CCL, CC chemokine ligand
C_T, threshold cycle
CXCR4, chemokine receptor 4
dMSO, dimethyl sulfoxide
dsDNA, double-stranded DNA
E, amplification efficiency
EDTA, ethylene-diamine-tetra-acetic acid
ERK, extracellular signal-regulated kinase
EST, expressed sequence tag
FBS, fetal bovine serum
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GDF5, growth differentiation factor 5
GM-CSF, granulocyte-macrophage colony-stimulating factor
GSC, Genome Sciences Centre
Hsp, heat-shock protein
ICE, p45 IL-1β-converting enzyme
IFNγ, interferon-gamma
IL-, interleukin-
IRAK-1, IL-1 receptor-associated kinase 1
JAK, Janus kinase
JNK, c-Jun N-terminal kinase
LBP, lipopolysaccharide-binding protein
LL-Z, LL-Z1640-2
LPS, lipopolysaccharide
MAP, mitogen-activated protein
MAPK, mitogen-activated protein kinase
MAPKK; MAP kinase kinase
MCP, monocyte chemotactic protein
MD-2, myeloid differentiation protein 2
MIME, Multiple Expectation Maximization for Motif Elicitations
MIP, macrophage inflammatory protein
MK2, mitogen-activated protein kinase-activated protein kinase-2
MyD88, myeloid differentiation factor 88
NCBI, National Center for Biotechnology Information
NF-κB, nuclear factor κB
NMR, nuclear magnetic resonance
p, probability chance
PAGE, polyacrylamide gel electrophoresis
PE, Perkin Elmer
PI3K, Phosphatidylinositol 3-kinase
PMA, phorbol 12-myristate 13-acetate
poly-A, polyadenylated
pre-mRNA, mRNA precursor
PTEN, phosphatase and tensin homologue
r, correlation coefficient
RAA, radicicol analog A
RANTES, regulated upon activation, normal T cell expressed and secreted
RefSeq, Reference Sequences
RPL32, ribosomal protein L32
RT-PCR, reverse-transcription polymerase chain reaction
SAGE, Serial Analysis of Gene Expression
SEM, standard error of the mean
SHIP, Src homology 2-containing inositol phosphatase
SK box, Shaw-Kamen box (AUUUA)
SNAPC2, small nuclear RNA activating complex, polypeptide 2, 45kDa
STATα, signal transducers and activators of transcription alpha
TAB1, TAK binding protein 1
TAK1, transforming growth factor β-activated kinase-1
TGF-β, transforming growth factor beta
TLR4, Toll-like receptor 4
Tm, melting temperature
TNF-α, tumor necrosis factor alpha
TRAF6, TNF receptor-associated factor 6
UV, ultraviolet
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Publications

Conference Proceedings

Mak I, Liu L, Kastelic T, Ling V
The Effect of RAA on mRNA Degradation in THP-1 Human Leukemic Monocytes: Potential for Cancer Treatment

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The Effect of RAA on mRNA Degradation in THP-1 Human Leukemic Monocytes: Potential for Cancer Treatment
1 Introduction

1.1 mRNA Stability

The development and progression of cancers are often associated with specific genetic changes. Some of these changes directly influence mRNA expression, which could in turn affect protein production. Eventually, the integrity and behavior of cells would be out of balance, resulting in an unrestrained growth. This research project will consider specific mRNA stability upon treatment with radicicol analog A (RAA) which was found to have a potent destabilizing effect on a small number of mRNAs (64).

mRNA expression in mammalian cells is highly regulated at both the transcriptional and post-transcriptional levels (33). The frequency of transcription is under the control of promoters, inducible transcription factors, and other regulating elements [reviewed in (59)]. Once pre-mRNA (an mRNA precursor) has been transcribed in the nucleus by RNA polymerase II, substantial post-transcriptional modifications must occur before the mRNA is ready for export to the translation machinery in the cytoplasm [reviewed in (37)].

1.1.1 Post-Transcriptional Regulation of mRNA Degradation

The dynamics of post-transcriptional processing introduces variations in mRNA and protein diversity, with multiple isoforms generated from single genes (11, 42). Processing of RNA precursors to their mature form, which may involve capping, editing, splicing, and polyadenylation, often occurs co-transcriptionally to ameliorate efficiency and specificity [reviewed in (5)]. All these post-transcriptional modifications would contribute to the mRNA stability / half-life.
Although the steady-state level of mRNA is a function of its rate of synthesis at a given time, it is controlled largely by its rate of degradation [reviewed in (122)]. mRNA decay is an important mechanism to regulate mRNA steady-state level (122). However, how an individual mRNA decay rate is controlled at the post-transcriptional level is still poorly understood (12). mRNA degradation is not a default nonspecific process where all mRNAs are degraded in the same fashion, but rather a tightly regulated process that determines the stability of an individual mRNA (18). The regulation of mRNA turnover involves specific cis-acting elements, trans-acting factors, and the activation of nucleases and other complexes [reviewed in (12)].

1.1.2 cis-Acting Elements

Post-transcriptional regulation is especially critical for modulating the turnover and translation rates of labile mRNAs encoding cytokines and proto-oncogenes (71), which transduce rapid and discrete cellular responses toward stimulation, differentiation or stress. This complex regulatory mechanism usually employs cis-acting elements, which are often, but not exclusively, located in the 3’ untranslated region (UTR) of mRNA sequences (12). Binding to the recognition sites of cis-acting elements, trans-acting factors would then initiate an assembly of the degradation complex with the exosome (26, 84) / nucleases (106, 122, 159) / ubiquitin (77, 78). Many recent studies suggest that trans-acting ARE-specific mRNA binding proteins, together with cis-elements, are responsible for regulating mRNA decay [reviewed in (12)]. Among numerous cis-elements identified, the most-studied and best-characterized regulatory cis-element to date is the AU-rich element (ARE) (139).
1.1.3 AU-Rich Element – Shaw Kamen box

Sequence analyses of the 3' UTR of labile mammalian mRNAs revealed that AU-rich regions usually contain at least one pentamer AUUUA and an AU-rich stretch of 50–150 nucleotides within the 3' UTRs (2). Shaw and Kamen's classic paper on ARE (139) showed that introducing a 51 nucleotide AU-rich sequence from human granulocyte-macrophage colony-stimulating factor (GM-CSF) into the 3' UTR of the rabbit β-globin mRNA markedly destabilized the otherwise highly stable β-globin mRNA in vivo. Since this AUUUA motif was first identified as a destabilizing element by Shaw and Kamen, this pentamer was named the Shaw Kamen (SK) box / sequence.

A variety of labile mRNAs, such as those encoding cytokines (45), lymphokines (15), inflammatory mediators (69), proto-oncogenes (107), and growth factors (149), are regulated through SK box-mediated mRNA turnover. The SK box-dependent mRNA degradation pathway allows a greater degree of flexibility in mRNA steady-state level for rapid cellular response to changes in environment or state of growth. Over-expression of these labile mRNAs due to dysregulation of mRNA stability is a genetic hallmark of cancer (56). For example, aberrant stabilization of IL8 mRNA has been observed in abnormal cell proliferation and malignant transformation (154). Loss of destabilizing AREs resulted in the stabilization of IL8 mRNA with lower degradation rates (175). Similarly, over-expression of an AU-rich binding factor, p37 AUFI (127), also promoted destabilization of mRNAs containing SK boxes. However, no literature published to date reports about modulation of SK box-containing mRNA degradation at the genomic DNA level.
The number of SK boxes and their organization are important in determining mRNA stability (2, 28, 176). AREs are highly diverse: each mRNA has a specific pattern of SK motifs which are similar to the conserved consensus but not exactly identical (107). Nonetheless, the presence of an ARE in the 3' UTR does not necessarily confer rapid mRNA decay. The half-life of some relatively stable mRNAs (e.g. luteinizing hormone receptor) was not affected by the removal of AUUUA motifs (98). Besides the SK box, the presence of a few copies of the nonamer UUAUUUA(U/A)(U/A), together with U-rich stretches, was a more potent destabilizer than a single SK box in certain models (75, 182).

1.1.4 Classic ARE Classification

Studies by Chen and Shyu (27) have shown that the number and organization of SK boxes are crucial in determining mRNA stability. They classified AREs into three categories based on distinct sequence features and decay characteristics (27, 107). Class I AREs, found in mRNAs like c-fos and c-myc, contain 1 to 3 scattered SK boxes coupled with nearby U-rich sequences (Table 1.1). Class II AREs, only found in cytokine mRNAs such as GM-CSF, interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), contain multiple overlapping copies (5 to 8 copies) of SK boxes (Table 1.1). Class III AREs, such as the one in c-jun mRNA, do not contain any SK box, but a long continuous U-rich region, and possibly other unknown features for their destabilizing function (Table 1.1).
<table>
<thead>
<tr>
<th>ARE Class</th>
<th>Sequence Features</th>
<th>Examples</th>
<th>Decay Kinetics</th>
<th>Mechanism of Deadenylation Reaction</th>
<th>Sensitivity to Actinomycin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 to 3 scattered copies of the pentamer AUUUA coupled with nearby U-rich regions</td>
<td>c-fos, c-myc</td>
<td>Biphasic decay, synchronous poly-A shortening</td>
<td>Distributive nucleolytic action; decay intermediate with poly-A tails of 30-60 nt</td>
<td>Sensitive</td>
</tr>
<tr>
<td>II</td>
<td>At least 2 overlapping copies of the nonamer UUAUUUA(A/U)(A/U) in U-rich regions</td>
<td>GM-CSF, TNF-α</td>
<td>Biphasic decay, asynchronous poly-A shortening</td>
<td>Processive nucleolytic action; decay intermediate missing poly-A tails</td>
<td>Sensitive</td>
</tr>
<tr>
<td>III</td>
<td>U-rich stretches without any AUUUA motif</td>
<td>c-jun</td>
<td>Biphasic decay, synchronous poly-A shortening</td>
<td>Distributive nucleolytic action; decay intermediate with poly-A tails of 30-60 nt</td>
<td>Insensitive</td>
</tr>
</tbody>
</table>

Table 1.1. Sequence features and functional properties of the ARE classes, adapted from Chen and Shyu (29).

mRNA turnover is mediated by AREs through two kinds of decay kinetics mechanism: (a) asynchronous deadenylation which results in an accumulation of non-poly-A intermediates in a processive fashion, (b) synchronous deadenylation which leaves behind decay intermediates with poly-A tails of 30 - 60 nucleotides in a distributive fashion. All three classes of AREs showed biphasic decay: after the specific deadenylation, the decay intermediate with the transcribed portion of the mRNA was degraded with first-order kinetics. ActD was found to block the rapid decay directed by both class I and class II AREs as demonstrated in β-globin mRNA carrying either the c-fos and GM-CSF AREs, but have little effect on β-globin mRNA carrying c-jun ARE, a class III ARE.
Generally, mRNA turnover is mediated by AREs through two mechanisms with different decay kinetics (27). First, asynchronous deadenylation, found only in Class II AREs, resulted in an accumulation of non-polyadenylated (non-poly-A) intermediates in a processive fashion. In contrast, synchronous deadenylation in Class I and III AREs directed a synchronous poly-A shortening, which left behind decay intermediates with poly-A tails of 30 - 60 nucleotides in a distributive fashion. However, all three classes of AREs display biphasic decay. The biphasic decay pattern demonstrated a two-step mechanism. First, a lag period when the poly-A tail was deadenylated. Then, the mRNA body was rapidly degraded with first-order kinetics (27). Intriguingly, actinomycin D (ActD), a transcription inhibitor extensively used in mRNA turnover studies, was found to block the rapid decay of synthetic β-globin mRNA carrying either the c-fos (class I) or GM-CSF (class II) AREs, but have little effect on β-globin mRNA carrying c-jun (class III) ARE (29, 107). These distinct features and properties of AREs again signified that different classes of AREs are differentially regulated through interaction with various trans-acting factors, such as ARE-binding proteins, depending on cell types and stimulations.

1.1.5 ARED

Besides the ARE classification of Chen and Shyu, an ARE-mRNA database (ARED) was established by Khabar et al. (9, 10, 66) in 2001. With a bioinformatics approach, the ARED documented the location of AREs in various human mRNA sequences. Initially, a database, which contained mRNAs encoding functionally diverse proteins from several disease states and important biological processes, was analyzed with Multiple Expectation Maximization for Motif Elicitations (MEME). MEME is an algorithmic program that analyses short
ungapped consensus sequences within a group of known human ARE-containing full-length mRNAs. MEME computed a derivation of a 13-base pair (bp) ARE pattern, WWWUAAUUAUWWW. This pattern was statistically validated for the specificity towards the 3' UTR and non-coding region. Subsequently, this 13-bp motif was used by the FindPattern analysis to scan through all non-redundant full-length human sequences extracted from GenBank in order to determine a valid and reliable list of ARE-containing mRNAs in ARED. Even though the full repertoire of ARE-containing mRNAs is still unknown, over 950 ARE-containing mRNAs from the human genome were compiled in ARED.

Cluster analysis categorized mRNAs into five groups using derivations of the 13-bp ARE pattern (9). As shown in Table 1.2, Group 1 - 4 contain five, four, three and two SK box (AUUUA) repeats, respectively, whereas Group 5 contains only one SK box within the 13-bp pattern. Clustering was performed with reduced stringency that balanced both sensitivity and specificity of selection. For example, Group 1 contained not only exactly five or more overlapping SK boxes, but also those with 10 % ambiguity and mismatch. As verified by a phylogenetic tree relationship, this analysis showed that the lower the number of ARE motifs in a group, the higher the number of sequences included, and the more functionally diverse the corresponding ARE-containing genes.
<table>
<thead>
<tr>
<th>ARED Clustering Group</th>
<th># of SK boxes in the ARE stretches with 10% ambiguity</th>
<th>ARE mRNA sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>AUUUAUUUAUUUAUUUAUUUA</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>AUUUAUUUAUUUAUUUA</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>WAUUAUUUAUUUAUUAW</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>WWUAUUAUUUAUUAW</td>
</tr>
<tr>
<td>V</td>
<td>1</td>
<td>WWWWAUUAUWW</td>
</tr>
</tbody>
</table>

Table 1.2. Sequence features of the clustering groups in ARED adapted from Bakheet et al (9).

10% ambiguity implies that 10% mismatch in the mRNA sequence is tolerated. W indicates any nucleotide (A, U, C, or G). ARE motifs in ARED clustering groups were computationally generated from a list of labile ARE-containing mRNAs, and matched against GenBank (2001, Release 113).
1.1.6 Antisense mRNA

Antisense mRNA is the complementary strand to the coding sequence of endogenous mRNA. The biological role of this novel transcriptional phenomenon may affect a variety of processes regulating gene expression (85). Naturally occurring antisense RNA could regulate expression of the sense transcript through interfering with mRNA transcription, maturation, stability, or translation (85). Introducing a transgene coding for antisense mRNA is a strategy used to block expression of a gene of interest (134). The double stranded RNA formed is known to cause RNA interference in eukaryotes (134). While past experiments have demonstrated a practical value of this approach for silencing eukaryotic gene expression (171), the molecular mechanisms underlying such regulation in eukaryotes remain unclear.

1.2 Radicicol Analog A

RAA (Fig. 1.1.B), a radicicol-related macrocyclic nonaketide compound owned by Novation Pharmaceuticals Inc., was derived from the fungal strain F/87-2509.04 (64). RAA was reported to reduce rapidly the mRNA expression level of a number of inflammatory cytokines in interferon-gamma (IFNγ) / lipopolysaccharide (LPS)-treated human monocytic leukemic THP-1 cells (64). mRNAs of these cytokines, including IL-1β, TNF-α, and IL-6, all contained SK boxes. In the study of Kastelic et al. (64), IL-1β mRNA, down-regulated by RAA, showed sustained mRNA expression upon RAA treatment when a portion of the 3' UTR that contained overlapping SK boxes was removed. Even though only 16 mRNAs were studied, just 10 mRNAs that contained at least one SK box were affected by RAA. Moreover, treatment with RAA was found to inhibit tyrosine phosphorylation of proteins
which were differentially expressed or differentially modified by a tyrosine kinase upon IFNγ/LPS stimulation (64).

In another study by Cheneval et al. (32), treatment with RAA resulted in a marked reduction in release of pro-IL-1β, as well as preventing the activation of p45 IL-1β-converting enzyme (ICE) in THP-1 cells stimulated with IFNγ/LPS. The pro-IL-1β cleavage by active ICE was inhibited. However, RAA did not block mature ICE activity.

In studies by Takehana et al. (157), RAA was found to inhibit AP-1-dependent but not NF-κB- nor glucocorticoid-dependent transcriptional activity in cell-based β-galactosidase reporter gene assays using phorbol 12-myristate 13-acetate (PMA)-, IL-1β-, and dexamethasone- induced HEK293 cells, respectively. AP-1 is a heterodimeric transcription factor composed of Jun, Fos, or ATF subunits, and its activity is partially regulated by phosphorylating its components (6, 138). Transcription factor AP-1, but not NF-κB, is the target of the ERK 1/2 mitogen-activated protein (MAP) kinase transduction pathway (1). TNF-α transcription was modulated by AP-1 binding to the promoter region of TNF-α gene (163). Because RAA suppressed AP-1 activity without suppressing the phosphorylation of c-Jun or ATF-2 (157), it is possible that the drug mediated its effects through the inhibition of transcriptional coactivators or the inhibition of phosphorylation of subunits other than c-Jun or ATF-2. Nonetheless, relatively little is known about the mechanism of action of RAA on mRNA destabilization.
Figure 1.1. Chemical structures of (A) radicicol, (B) RAA, and (C) LL-Z.
Figure 1.2. Chemical structure of geldanamycin.
1.2.1 Radicicol

Radicicol (Fig. 1.1.A), also known as monorden, is a macrocyclic lactone originally isolated from the fungus, *Monosporium bonorden* [reviewed in (74)]. RAA may retain some chemical properties of radicicol due to similar structures. Radicicol is a classic inhibitor of protein tyrosine kinases (74). It has been shown to suppress transformation by *src* and *ras* oncogenes (67, 68, 73, 111, 145). While being relatively inactive against a wide range of serine (Ser) / threonine (Thr) kinases (e.g. PKC, PKA) of the mitogen activated pathway (74), radicicol potently inhibited the receptor tyrosine kinase p60 v-src, which leads to cell arrest in G1 and G2 (140, 141).

Moreover, radicicol shares a molecular mode of action with the benzoquinone ansamycin class of antibiotics, such as geldanamycin (Fig. 1.2), even though the chemical structure of radicicol is very different from geldanamycin (120, 121, 126, 132). Geldanamycin does not inhibit tyrosine kinase directly, but modulates its activity via the heat-shock protein (Hsp) 90 molecular chaperone (121, 131). Similarly, radicicol exerts its anti-proliferative properties through disrupting Hsp90-mediated protein kinase activation (3, 82, 120). Hsp90 is a folding and scaffolding protein that binds the *src* family kinases and the Ser / Thr kinase, Raf-1, among other proteins (53, 114, 131, 137). In an ATP-dependent manner, Hsp90 plays an essential role in the activation of a range of proteins involved in signal transduction and cell cycle regulation (53, 132, 181). Treatment of cells with radicicol dissociates Hsp90 from Raf-1, leading to destabilization and degradation of Raf-1 (13, 65, 180). The depletion of Raf-1 would result in the inhibition of the MEK / ERK MAP kinase pathway (68, 109). Due to radicicol's many biological properties and several apparent intracellular targets, the
elucidation of details relevant to the mechanism of radicicol's biological profile still remains an active area of study.

1.2.2 Radicicol-Related Compounds

LL-Z16402 (LL-Z) (Fig. 1.1.C), is another naturally occurring compound related to radicicol (36). The structure of LL-Z is similar to RAA, except that it is missing one methoxy group on the C1 of nonaketide, and the hydroxyl group on the C13 is in the opposite orientation, as shown in Figure 1.1. LL-Z selectively inhibits the activation of anisomycin-induced c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways at the MAP kinase kinase (MAPKK; a.k.a. MEK) level, but not the TNF-α-induced activation in HeLa cells (157). Despite its resemblance to LL-Z, RAA apparently did not show any influence on the activation of JNK / p38 MAP kinase pathways in the same system. Notwithstanding, p38 MAPK pathway is a potential candidate target of RAA because its downstream target, mitogen-activated protein kinase-activated protein kinase-2 (MK2), essentially regulates post-transcriptionally the LPS-induced expression of cytokines through AREs at the level of mRNA stability and translation (16, 99). Interestingly, LL-Z did not inhibit TNF-α-induced JNK activation, indicating that the inhibition of the JNK pathway by LL-Z was signal-specific (157).

Recently, LL-Z and a number of derivatives were found to inhibit specifically the catalytic activity of transforming growth factor β-activated kinase-1 (TAK1) MAPK kinase kinase (101). TAK1, together with its coactivator TAK binding protein 1 (TAB1), coupled the activation of NF-κB and JNK MAPK pathways, with the p38 MAPK pathway for mRNA
stabilization in HeLa cells (57). TAK1 also mediated the LPS-driven PKB / Akt survival pathway (80). Additionally, it is interesting to note that although LL-Z was claimed to inhibit TAK1 which regulated NF-κB pathway, the transcriptional activity of NF-κB was not inhibited (157).

In two other recent studies, LL-Z suppressed HgCl2-induced JNK / p38 activation in CCRF-CEM and NIH3T3 cells (92, 93, 153). From cDNA microarray experiments on the expression of stress response genes in cells exposed to CdCl2, treatment with LL-Z reduced the expression of Hsp70 markedly in NIH3T3 cells (153). Other JNK inhibitors could not achieve similar levels of inhibition nor subdue the same set of mRNAs. Nevertheless, mechanisms by which LL-Z down-regulated mRNA stability have not been reported in the published literature to date.

1.3 THP-1 leukemic monocytes

The human monocytic leukemia cell line, THP-1 was established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (AML-M5) (162). THP-1 cells behave like monocytes (162). After differentiation, THP 1 cells are metabolically and morphologically similar to monocyte-derived macrophages (8). THP-1 cells have a number of genetic aberrations, including the t(9;11), associated with AML M5 (34). THP-1 monocytes can be induced to differentiate into macrophages by treatment with IFNγ / LPS or PMA in vitro (166). The THP-1 cell line provides a valuable cell culture model for investigating mechanisms involved in monocyte / macrophage differentiation and activation. (115, 178). IFNγ / LPS stimulated THP-1 cells were the primary model used in this study
because they expressed a high level of SK box-containing mRNAs, which encode cytokines and oncogenes.

1.3.1 IFNγ Signal Transduction Pathways

IFNγ is usually secreted from CD4+ Th1 cells, CD8 cells, γ/δ T cells, and activated NK cells (25, 41, 123, 133, 173). It plays a role in activating lymphocytes to enhance anti-microbial and anti-tumor effects (14). In addition, it plays a role in regulating the proliferation, differentiation, and response of lymphocyte subsets (63, 104). Together with LPS, treatment with IFNγ also triggers an increased secretion of macrophage-derived nitric oxide (136).

IFNγ receptors are found in high amounts on the cellular surface of many different cell types, such as T cells, B cells, monocytes / macrophages, NK cells, and fibroblasts. Signaling takes place through an IFNγ receptor complex consisting of two alpha chains (Type I receptor) and two beta chains (Type 2 receptor) (112). The receptor consists of an alpha subunit to which IFNγ binds, and a beta subunit necessary for signal transduction (19). The alpha subunit is associated with Janus kinase 1 (JAK1) and the beta subunit is associated with JAK2 (147). After IFNγ binds to its receptor, the alpha subunit, as well as JAK1 and JAK2, are tyrosine phosphorylated. JAK1 then phosphorylates a tyrosine residue on Signal Transducers and Activators of Transcription alpha (STATα) (62). STATα is a convergent point for immunological stimuli in a macrophage pro-inflammatory response (148), and the strength of signal through the IFNγ receptor may influence immune responsiveness (135). After phosphorylation, two STATα molecules dimerize via the interaction of their SH2 domains.
This dimer, IFNγ activator factor (GAF), is then translocated into the nucleus and binds to IFNγ activator sequence activation sites of IFNγ-inducible genes (117), resulting in an elevated transcription of IFNγ and IFNγ-inducible genes, encoding for proteins with cytokine and growth suppressive activities (105).

1.3.2 Lipopolysaccharide and Cytokine Expression

Similar to peripheral blood monocytes, THP-1 cells respond to stimulation, such as with LPS or PMA, by rapidly increasing gene expression of specific cytokines (39, 52). LPS is a major component of the outer membrane of Gram-negative bacteria (47).

The production of cytokines in monocytes / macrophages is triggered by interaction with cellular defense mechanisms, micro-organisms, microbial products (e.g. LPS), and endogenous factors including cytokines themselves (165). Many studies have shown that these cytokines play a pivotal role in a variety of pathophysiologic responses, including inflammation, fever, coagulant activity, bacterial septic shock, and death, although the precise function of each cytokine individually remains to be elucidated [reviewed in (54)]. In response to LPS, monocytes produce large quantities of pro-inflammatory cytokines (7, 51, 156). Activating cytokines (IL-1, IL-3, IL-6, IL-8, IL-12, TNF-α, GM-CSF, IFNγ) up-regulate the production of cytokines in macrophages (71) while deactivating cytokines (IL-4, IL-10, IL-13, TGF-β) inhibit it (50). In addition, all these cytokines can modulate many macrophage functions and cell surface marker expression. Other cytokines, such as the CC chemokine ligand (CCL) family: CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL3 /
4 (MIP-1), and CCL5 (RANTES), recruit circulating monocytes to tissues (61). It is worth noting that macrophages can be their own source of regulatory cytokines.

Most of the mRNAs studied that were down-regulated by treatment with RAA in THP-1 cells, were cytokines (64). The two most important cytokines secreted from monocytes / macrophages during LPS stimulation are TNF-α and IL-1β (4, 21, 100, 146, 164). IL-1β and TNF-α have similar and broad ranges of physiological actions in regulating local and systemic immune responses. In addition, cytokines mRNA usually contain at least one SK box in the 3' UTR (45, 151, 174). Yet, some cytokines, such as CCL5 / RANTES, do not have any SK boxes at the 3' UTR.

1.3.3 LPS Signaling Pathways

As shown in Figure 1.3, LPS-induced activation of monocytes / macrophages involves the receptor complex of CD14 / Toll-like receptor 4 (TLR4) / myeloid differentiation protein (MD)-2 (79, 161, 172). Assembly and activation of this complex on the plasma membrane result in the membrane translocation of the intracellular adaptor protein myeloid differentiation factor (MyD) 88, followed sequentially by the intracellular activation of IL-1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6) (60, 125, 155, 179).

Upon LPS stimulation, several signaling pathways in monocytes / macrophages are turned on. The ERK 1 / 2, p38, JNK MAP kinase, and IKK / NF-κB signaling pathways, downstream of the stimulated receptor, become activated, which in turn promotes gene
transcription via transcription factors such as NF-κB and AP-1 (47, 158) (Fig. 1.3). Upon activation of the NF-κB transcription factor, NFKBIA dissociates from NF-κB and is degraded by proteasomes (17). NF-κB translocates to the nucleus where it can activate the transcription of a large number of immuno-relevant genes. Although the MAP kinase pathways are important in regulating mRNA stability (95, 124), the signaling events that bridge receptor stimulation to the activation of MAP kinases, are not fully understood.

Phosphatidylinositol 3-kinase (PI3K) is also activated in response to LPS stimulation as a negative control (Fig. 1.3) (38). The inhibitory effect of PI3K is mediated via the Ser / Thr kinase, Akt. Activation of the PI3K-Akt pathway inactivates MAP kinases and the NF-κB pathways by phosphorylation of Raf-1, I-κB kinase, GSK-3β, and other upstream kinases, such as MEKK3 (48). SHIP, Src homology 2-containing inositol phosphatase, is tyrosine phosphorylated upon LPS stimulation. Activation of SHIP would inhibit the PI3K pathway, as shown in Figure 1.3 (38, 144). PTEN, phosphatase and tensin homologue, was found to have similar effects on LPS-TLR4 signaling by down-regulating Akt (23).
Figure 1.3. LPS stimulation in monocytes activates signaling pathways and transcription factors for cytokine production.
LPS binds to the serum protein LBP and is transferred to CD14 at the cell surface. LPS then interacts with the signaling receptor complex: TLR4, MD-2, MyD88, TRAM, IRAK, and TRAF6. LPS stimulates the activation of various MAPK pathways, including the ERK, JNK, and p38 pathways. These pathways directly or indirectly phosphorylate and activate various transcription factors, including Elk-1, c-Jun, c-Fos, and ATF-1. In addition, LPS activates the IKK pathway via MyD88, IRAK, and TRAF6. Subsequent degradation of IKβ permits nuclear translocation of NFκB / Rel complexes, such as p50 / p65. The PI3K-Akt pathway phosphorylates and activates p65 via an unknown kinase. All these pathways modify the expression of downstream transcription factors, such as AP-1 and NF-κB, through which cytokine mRNA transcription is regulated.

This diagram is modified based on the work of Guha et al. (47, 48)
Taking into account the discovery of the importance of TLR4 in LPS signaling, an LPS signaling complex of receptors was recognized recently as shown in Figure 1.4 (161). Using affinity chromatography and peptide-mass fingerprinting, Triantafilou et al. (160) identified a structurally heterogeneous complex of many receptors that could bind LPS. The LPS activation cluster comprised Hsp70 and Hsp90 (their importance has been stated in section 1.2.1 and 1.2.2), chemokine receptor 4 (CXCR4), CD11 / CD18 / CD55, and growth differentiation factor 5 (GDF5). The composition of the receptor cluster and the stoichiometry of the receptors determine the mechanism for recognizing and regulating cell signaling in response to a broad range of microbial pathogens. The recruitment of different receptors is triggered by different stimulations.
Figure 1.4. Hypothetical model for the immune recognition of LPS from Triantafilou et al. (161).

(a) Lipopolysaccharide-binding protein (LBP) binds and catalyses the transfer of LPS to membrane-bound CD14.

(b) Signaling molecules are recruited to the site of CD14–LPS ligation. LPS is released from CD14 in the lipid bilayer, and the intercalated LPS binds to a complex of receptors: CXCR4, Hsp 70/90, GDF5, and possibly CD55.

(c) Signal transducing molecules, such as TLR4 complexed with MD-2, Toll-like receptors (TLRs) and / or integrins CD11 or CD18 are further recruited into the activation cluster, triggering multiple signaling cascades.
1.4 Serial Analysis of Gene Expression

In the work of Kastelic et al. (64), RAA appeared to down-regulate all 10 SK box-containing mRNAs from IFNγ / LPS stimulated THP-1 cells. It was important to study the effect of RAA on the entire transcriptome expression in order to characterize and generalize the effect of RAA to all expressed genes. To this end, Serial Analysis of Gene Expression (SAGE) (167) was used to assay the transcriptome-wide gene expression profile of human THP-1 leukemic monocytes treated with RAA.

SAGE is a powerful, efficient, and comprehensive approach for analyzing gene expression profiles quantitatively. SAGE is mainly based on two principles: (i) representation of mRNAs (cDNAs) by short sequence tags, and (ii) concatenation of these tags for cloning to allow their efficient sequencing analysis (177). It is chosen over other large-scale high-throughput approaches, such as microarrays of cDNAs or oligonucleotides on solid matrices, because the readout in these techniques relies on comparative hybridization with labeled probes (i.e., cDNAs) of known genes. Because the hybridization conditions and data processing vary, it is difficult to compare expression array data between laboratories (46, 152).

SAGE, on the other hand, measures the proportional transcriptome expression levels through a tag counting approach, allowing data to be obtained and compared from different samples analyzed independently. This method is used not only to characterize quantitative information on the abundance of known transcripts, but also to identify unknown expressed genes. Various groups have demonstrated the utility of SAGE for discovery of unknown
genes and pathways, for biomarker identification, and for gene expression profiling of numerous disease and normal conditions in multiple species (20, 31, 51, 58, 94). Another elegant feature of the SAGE process is that the position and orientation of a SAGE tag on an mRNA molecule can be accurately determined. This feature of SAGE analysis not only allows validation of tag identity, but also has the unexpected added benefit of detecting antisense transcription.

1.4.1 Preparation of SAGE Libraries

SAGE technology is based on the generation of representative tags of short nucleotide sequences (ShortSAGE, 14 bp, or LongSAGE, 21 bp) at a unique position within each species of mRNA (Fig. 1.5) (167, 177). Poly-A RNAs are extracted and transcribed into double stranded cDNAs, using biotinylated oligo-dT coated beads as a primer. Digestion with a four-base recognizing Anchoring Enzyme (usually NlaIII, a type II restriction enzyme) results in cDNA fragments with a length of 256 bp on average. The restriction sites of the Anchoring Enzyme are at a uniquely defined position within each cDNA, which is important for the ultimate identification of the corresponding genes. The biotinylated 3'-most fragments then are isolated using paramagnetic streptavidin coated beads.

The fragments subsequently are divided in half and ligated to two different linkers. Each linker is designed to contain a recognition motif for the Tagging Enzyme (usually BsmFI, a type IIS restriction endonuclease) that cuts at the restriction site close to the Anchoring Enzyme overhang. By digesting these bound linker-cDNA sequences with the Tagging Enzyme, fragments consisting of linker and an adhering short cDNA sequence (a SAGE tag)
are released from the streptavidin beads. The staggered ends of isolated linker tags are blunt-ended with the Klenow fragment of T4 DNA polymerase I.

Since the 5'-ends of the linkers are blocked by an amino group, only the mRNA-derived termini are able to be ligated in a tail-to-tail orientation when the two sets of linker tags are mixed. The constructs are amplified by PCR using primers specific to the linkers. Digesting these constructs with the Anchoring Enzyme finally releases the ditags, which are separated by polyacrylamide gel electrophoresis (PAGE). Ditag fragments flanked at both ends with NlaIII cohesive terminus are isolated and ligated to obtain concatemers. Highly concatenated products are recovered by PAGE, and cloned into a plasmid vector for sequencing. The sequences obtained are compared to genes from various genome databases in order to identify the tags.

In the SAGE community, libraries smaller than the arbitrary limit of 50,000 tags are considered "small" (168). Small libraries yielded similar overall results to large libraries, as shown in the studies of Vencio et al. (168). These libraries are not always outliers from biological sampling, but are samples like any other large libraries. Nevertheless, it was suggested that one can use the "small" libraries, jointly with "non-small" ones to include more biological variability because in most libraries, the majority of SAGE tags is present at low levels (tag number ≤ 15) (90).
Figure 1.5. Schematic depiction of the SAGE procedure.
As template for cDNA synthesis, poly-A RNAs are isolated or directly captured from cell lysates using oligo-dT-coated beads. The anchoring enzyme most frequently used is NlaIII. The CATG 5' overhangs are used to ligate specific linkers. These linkers have built in recognition motif for the tagging enzyme, usually BsmFI. This enzyme cuts cDNA from the restriction site, releasing the tags with the linkers from the cDNAs. These tags are ligated tail to tail and amplified by PCR generating ditags. After purification, ditags are released from the linkers, concatenated, cloned, and sequenced. The abundance of each tag in the cloned products is directly proportional to the abundance of the corresponding transcript in the original sample. The relative abundance of a transcript is calculated by dividing each specific tag count by the total number of tags sequenced.

This diagram is taken from [http://sciencepark.mdanderson.org/ggeg/SAGE_technique.htm](http://sciencepark.mdanderson.org/ggeg/SAGE_technique.htm), which is adapted from the original article of Velculescu et al. (167)
1.5 Real-Time Reverse Transcription Polymerase Chain Reaction

As the interpretation of SAGE can often be inaccurate or misleading in samples with low gene expression (83), SAGE results should be followed up with a complementary method to quantify gene expression. The most popular method to quantify gene expression is the real-time reverse transcription polymerase chain reaction (RT-PCR) method. Fluorescence intensity is measured during each cycle, and the amount of fluorescence is proportional to the amount of PCR product. In real-time RT-PCR, reactions are characterized by the first detected fractional cycle number (Threshold Cycle, C_T) at which the amount of amplified target reaches a fixed threshold (87), rather than the amount of target PCR product accumulated after a fixed number of cycles in traditional PCR (130). This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. The higher the starting copy number of the nucleic acid target is, the sooner a significant increase in fluorescence is observed.

The real-time RT-PCR technique is exquisitely sensitive, permitting analysis of gene expression from very small amounts of RNA (even at the level of the content of a single cell) (96). This feature complements the inability to detect low-expressed mRNAs in SAGE libraries when the total tags sequenced are below 50,000 tags. With an increased dynamic range of detection, less than a 2-fold change in expression is detectable (97). Moreover, a large number of reactions (up to 384 reactions) from various samples and genes can be analyzed in the same experiment. This gives the investigator a measure of flexibility and sensitivity unavailable in other approaches, such as Northern blot, RNA dot blot, RNAse protection assays, and in situ hybridization. Data from real-time RT-PCR gives an
immediate appreciation of the PCR kinetics occurring within the reaction tube, and, in addition, gives an instantaneous visual representation of the amount of PCR product present following each cycle (118).

With the ability to collect data in the exponential growth phase, the power of real-time RT-PCR has been expanded into many applications, such as viral quantitation, quantitation of gene expression, high-throughput assay verification, drug therapy efficacy, DNA damage measurement, quality control validation, pathogen detection, and genotyping, etc. (102). Real-time RT-PCR was used in this project to follow up on the interesting preliminary expression data from the SAGE libraries through validation and quantification, as well as to determine the half-lives of various mRNAs.

1.5.1 SYBR Green I Dye

All real-time PCR systems rely upon the detection and quantitation of a fluorescent reporter. In the simplest and most economical format, that reporter is the double-strand DNA (dsDNA)-specific dye SYBR Green I. SYBR Green I binds in the minor-groove of dsDNA, and upon excitation, emits light (86). The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively (91). The two most popular alternatives to SYBR Green I are TaqMan and molecular beacons, both of which are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantitation (102). All these dyes were specifically developed for real-time RT-PCR because the fluorescence signal increases when they bind to dsDNA, but none of these dyes inhibits PCR (55, 143).
The advantage of SYBR Green I chemistry over other hybridization probes is that the SYBR Green I dye is inexpensive, easy to use, and sensitive, even though it detects all dsDNA, including primer-dimers and other non-specific reaction products, which could result in an overestimation of the target concentration (169). A well-optimized reaction that raises the PCR specificity is, therefore, essential for accurate results when using SYBR Green I dye. For single PCR product reactions with well designed primers, SYBR Green I works well, with false background appearing only in very late cycles (113). Therefore, SYBR Green I dye, which is compatible for use with any real-time thermocycler, was chosen for this project.

1.5.2 Real-Time RT-PCR Quantification Method

RT-PCR usually takes place in a one-stage or two-stage reaction, and this project was operated entirely in two-stage RT-PCR mode. For analyzing gene expression levels, isolated mRNA was first transcribed into cDNA using a reverse transcriptase, together with either oligo-dT primers, random oligomers, or gene-specific primers. Following the RT reaction, cDNA was amplified by the PCR method. The PCR method is generally a three-step process: denaturation, annealing, and elongation steps. Temperatures used during these steps vary, and are subject to a number of considerations that should be determined empirically (116). The number of cycles depends on the amount of target present and also the efficiency of the reaction (43). By selecting the threshold cycle within the log-linear phase for all samples, it is possible to calculate the actual amount of initial starting molecules since the fluorescence intensity is directly proportional to the amount of PCR product in the exponential phase (87).
The amount of the target gene products can be expressed as either Absolute or Relative quantification (these two terms are designated for real-time RT-PCR quantification methods). Absolute quantification determines the absolute amount of target expressed as copy number or concentration (22, 72), whereas relative quantification determines the ratio between the amount of target and a stably expressed endogenous reference / housekeeping gene (88). This normalized value can then be used to compare, for example, differential gene expression among various samples or conditions (129).

For calculating the absolute quantification, a standard curve, which plots the log of the amount of standard against CT values, is generated using a dilution series of five or more different concentrations of the standards, as shown in Figure 2.1 A (in Materials and Methods section) (170). The amount of unknown target should fall within the range of standard amount tested. Relative quantification also requires a standard curve to determine the amplification efficiency of real-time RT-PCR (40). Due to differences in PCR efficiency, the resulting standard curves would not be parallel. The differences in CT values of the target and the reference would not be constant because the template amounts vary (49, 87, 97). Both relative and absolute quantification methods were employed in this project.
1.6 **Hypothesis**

Some tumors develop the capacity to stabilize labile proto-oncogene mRNAs, leading to protein overproduction and a predisposition to unrestrained growth. Restoring rapid decay to those mRNAs which exhibit prolonged mRNA half-life in disease states, would be a potential strategy for treating cancer and other diseases. RAA is a potent compound that appears to selectively destabilize a small number of proto-oncogene and cytokine mRNAs, which contain AREs, particularly SK boxes, in human THP-1 leukemic cells stimulated with IFNγ / LPS (64). If the observations reported by Kastelic *et al.* (64) extended to the entire transcriptome, we hypothesized that RAA would only down-regulate mRNAs containing common features, particularly SK boxes, and that the number and organization of SK boxes would predict the degree of RAA sensitivity. Thus, the objectives of this research project were:

(i) to determine whether RAA only down-regulates SK box-containing mRNAs;

(ii) to determine whether the number and organization of SK boxes in mRNAs correlate with sensitivity to RAA.

SAGE libraries prepared from IFNγ / LPS stimulated THP-1 cells in the presence or absence of 1 µM RAA had already been constructed by Ms. Lin Liu and Dr. Tania Kastelic, and were sequenced by GSC using DiscoverySpace software. Focus was placed on utilizing the SAGE data to determine and characterize the effect of RAA on mRNA down-regulation in the leukemic monocytic cell line, THP-1, at various stages of differentiation, as well as examining mRNA half-life.
In this project, some of the mRNAs seen to be down-regulated in SAGE libraries were assayed with the more sensitive real-time RT-PCR method. After profiling and patterning the fold change in mRNA expression of selected RAA down-regulated mRNAs of interest using real-time RT-PCR, the mRNA half-life of selected mRNAs at various stages of THP-1 differentiation was measured and analyzed. Finally, the degree of RAA sensitivity of these mRNAs were analyzed based on the number and organization of SK boxes, their gene expression patterns after differentiation, and their half-lives.
2 Materials and Methods

2.1 Cell Culture and Maintenance

THP-1 human leukemic monocytic cells were cultured in RPMI medium 1640 containing 2.05 mM L-glutamine (Cat. # 11875-119, Gibco, Burlington, Ontario) supplemented with HEPES (25 mM, Sigma, Oakville, Ontario), penicillin (50 U / mL, Gibco), streptomycin (50 µg / mL, Gibco), and 2-β-mercaptoethanol (βME, 50 µM, cell culture grade, Cat. # M7522, Sigma). Heat-inactivated fetal bovine serum (5 v / v %, FBS, Gibco) was added before use. The THP-1 cells were grown to a density of $5 \times 10^5$ cells / mL (at the log phase of growth) at 37 °C in a 95 % humidified incubator with 5 % CO₂. To stimulate the differentiation of monocytes into macrophages, THP-1 cells were treated with IFNγ (100 U / mL, Human, recombinant E. coli, Cat. # 1040596, Roche, Mississauga, Ontario) at t = -3 h. Three hours later (t = 0 h), LPS (5 µg / mL, Cat. # L8274, E. coli serotype O26:B6; Sigma) was added to stimulate the inflammatory response.

2.2 RAA Treatment

RAA (Novation Pharmaceuticals Inc., New Westminster, British Columbia) has an empirical formula: $\text{C}_{20}\text{H}_{24}\text{O}_8$, ((7S,12S,13S)-(9Z15E)-4,12,13-trihydroxy-1,2-dimethoxy-7-methyl-8,12,13,14-tetrahydro-7[H]-6-oxabenzocyclotetradecene-5,11-dione), and a molecular weight of 392.405 g / mole. After THP-1 cells were stimulated with LPS for 16 hours, RAA (1 µM) prepared in 100 % methanol (Sigma) or methanol alone was added (t = 16 h). Final methanol concentration in the medium was 0.1 %.
2.3 Time Course of Differentiation

THP-1 cells were cultured and differentiated as described in section 2.1. Total RNA from THP-1 cells, isolated at t = -3, 0, 1, 2, 4, 8, 12, 16, and 24 h, were used to examine the pattern of gene expression of mRNAs using the real-time RT-PCR method.

2.4 RNA Isolation and Reverse Transcription

Total RNA was isolated from THP-1 cells lysed with lysis buffer (Buffer RLT) containing βME using the RNeasy Midi Kit (Cat. # 75154, Qiagen, Mississauga, Ontario) according to the manufacturer’s instructions. Both supernatant and adherent portions were collected from plates for lysis. Total RNA was eluted into DEPC-treated water (0.1% diethylpyrocarbonate in Milli-Q water, autoclaved). The quantity and purity of total RNA were evaluated by the optical density at 260 nm and 280 nm using an ultraviolet (UV) spectrophotometer (Hitachi U-2000, Tokyo, Japan). The amount of total RNA was quantified using the absorbance at 260 nm (A260):

\[
\text{Concentration in } \mu\text{g/ml} = A_{260} \times 50 \text{ (weight per optical density) } \times \text{ dilution factor}
\]

The indication of the RNA purity was measured by the ratio between A260 to A280 (see Current Protocols in Molecular Biology). To ensure complete removal of DNA, isolated total RNA aliquots (1 μg) were treated with 1 μL of 10 × DNase I buffer (Invitrogen Life Technologies, Burlington, Ontario, Canada), 1 U (≈ 1 μL) of amplification-grade RNase-free DNase I (Invitrogen), and RNase-free water in a 10 μL total volume at 25°C for 15 min. The reaction was stopped with the addition of 1 μL of ethylene-diamine-tetra-acetic acid (25 mM, EDTA) (Invitrogen) at pH 8.0, and heating at 65°C for 10 min. The isolated mRNAs were reverse transcribed into cDNAs with the SuperScript II First-Strand Synthesis System (Cat. #
11904-018, Invitrogen) following the manufacturer’s instructions. mRNAs were reverse transcribed using oligo-dT.

2.5 PCR and PAGE

The PCR reaction was performed with 1:16 diluted cDNA template (2 μL) obtained from RNA (1 μg), 3.04 μL of each 5’- and 3’-primer (2 μM) for both the target and housekeeping genes, 0.6 μL of dNTP mix (10 mM, Invitrogen), 1 U of Taq DNA polymerase (made by Ling Liu from E. coli strain DH1, according to Current Protocols in Molecular Biology), 2.0 μL of 10 × PCR buffer (Invitrogen), in a total volume of 20 μL. Following an initial denaturation at 95 °C for 15 s, the amplification program on a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, Streetsville, Ontario) was set as follows: initial denaturation at 95 °C for 15 s, 35 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C (variable, depending on the melting temperature) for 30 s, and extension at 68 °C for 30 s. A final extension was at 68 °C for 5 min, followed by incubation at 4 °C.

The size of the amplified PCR product was visualized using PAGE. PAGE gels were cast using a reaction mixture: 8 % polyacrylamide (37.5:1 acrylamide:bis, BioRad, Mississauga, Ontario,), 1 × TAE buffer [50 × stock solution: 2 M Tris base (Sigma), 0.057 v / v % acetic acid (Sigma), and 0.1 M EDTA (Sigma), pH 7.5], 0.1 % filtered ammonium persulfate (APS, Gibco), and 10 μL TEMED (Gibco), in a total volume of 10 mL. PCR samples (20 μL) were loaded with 2 μL of 10 × DNA loading dye (bromophenol blue, xylene cyanol, and glycerol, as described in Current Protocols in Molecular Biology) into each well, and a 100 bp DNA ladder (Invitrogen) was included in each run. Gels were run at 100 volts for about 1 h at
room temperature until the blue dye was about 1 inch from the bottom edge of the gel. cDNA bands were detected by staining at room temperature for 15 min with 1:10 diluted SYBR gold (Molecular Probes, Burlington, Ontario) or ethidium bromide (EtBr, Sigma). Gels were photographed with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA).

2.6 cDNA Extraction

Desired cDNA bands from the polyacrylamide gel were cut out, and crushed by spinning in a spin column for 3 min at 13,000 rpm on a bench-top centrifuge (Eppendorf 5415 D, Brinkmann Instruments Inc., Westbury, NY). Crushed mixture was incubated in 167 μL of low salt TE buffer (Invitrogen) and 33 μL of ammonium acetate (7.5 M, Invitrogen) at 65 °C for 2 h. After the mixture was filtered through a filter column, the filtrate was extracted with 200 μL of phenol-chloroform (Invitrogen). The extract was incubated with 130 μL of ammonium acetate (7.5 M, Invitrogen), 3 μL of glycerol (Invitrogen), and 1 mL of cold 95% ethanol on dry ice (< -20 °C) for 50 min. The mixture was centrifuged at 13,000 rpm for 30 min at 4 °C, and then was washed twice with cold 70% ethanol at -20 °C. cDNA pellet was air dried and resuspended in 300 μL of RNase-free water. The quantity and purity of extracted cDNA were calculated from the absorbance, gauged by the optical density at 260 nm and 280 nm.

2.7 Data Mining on SAGE Libraries

Two SAGE libraries were prepared from total RNA isolated from THP-1 cells stimulated with IFNγ / LPS (t = 24 h) (as described in 2.1, in the absence of βME) treated at t = 16 h with RAA (1 μM) or methanol for 8 hours were constructed by Dr. Tania Kastelic and Ms.
Lin Liu, with the I-SAGE Kit (Cat. # T500001, Invitrogen) according to the manufacturer's instructions. The DNA sequencing was carried out at the Genome Sciences Centre (GSC) of the BC Cancer Agency (BCCA, Vancouver, British Columbia) by Jeff Stott and George Yang. Cells with or without RAA treatment were used to generate two libraries.

Data were analyzed with the DiscoverySpace software v3.1.2 (GSC), developed by Scott Zuyderduyn and the Gene Expression Bioinformatics team at GSC (http://www.bcgsc.ca/bioinfo/software/discoveryspace/). This software extracts tags from the sequences, and creates a report of each tag and its abundance level. It provides a graphical user interface for visualization, and analysis of the frequency and distribution of SAGE tags, together with the hypothetical identities of the tags through accessing multiple data sources, such as National Center for Biotechnology Information (NCBI) taxonomy and Online Mendelian Inheritance in Man (OMIM). In addition, the software eliminates all linker generated tags, tags with poly-A sequences, duplicate tags, and clones containing \( \leq 4 \) tags. The remaining tags constituted the pool of reliable tags. The DiscoverySpace software has also various built-in statistical analysis tools that calculate the relative likelihood that a difference would be seen by chance for an individual tag or an entire project. For calculating the statistical significance between samples, simulations were performed assuming the null hypothesis. The p-value (probability chance, \( p \)) of a statistical hypothesis test is the probability of getting a value of the test statistic as extreme as or more extreme than that observed by chance alone, if the null hypothesis is true. This is a relative probability of obtaining the observed differences due to random variation.
Subsequent analyses on the results provided by this software included: (i) identification of unique SAGE tags from the total SAGE tags; (ii) designation and verification of the potential tag-to-gene identity; (iii) determination of the ratio of unique SAGE tags that match to ARE-containing mRNAs, and (iv) determination of the ratio of unmatched unique SAGE tags. Investigation on the SAGE libraries acquired quantitative information on four types of transcripts: (a) tags identifying known genes; (b) tags identified from anonymous expressed sequences (i.e., EST clusters); (c) tags identifying transcripts from the antisense strand; and (d) tags identifying transcripts with no matches to any of the available databases.

Based on the 99.9% confidence level, tag-to-gene identities of down-regulated SAGE tags were assigned using: (1) DiscoverySpace software v3.1.2 accessing the UniGene and RefSeq database of NCBI; (2) the NCBI database through the BLAST sequence search tool v2.2.6 (http://www.ncbi.nlm.nih.gov/BLAST/); (3) SAGEmap of the NCBI (http://www.ncbi.nlm.nih.gov/SAGE/, Nov. 2003 version); and (4) SAGE Genie of the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/SAGE, Nov. 2003 version).

2.8 Primer Design
DNA and mRNA sequences of genes were obtained from GenBank Release 137 at the NCBI (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) for primer design. Once the exon / intron boundaries on mRNAs were located, forward and reverse primers were designed flanking the exon-exon junctions. Primers were chosen based on the primer analysis software Primer Express v2.0 (PE Applied Biosystems). To obtain the optimal specificity and performance in real-time RT-PCR, primers were required to have melting
temperature (Tm) between 56-60 °C, of length about 20-22 bp, and with penalty index less than 100. Primer pairs produced amplicons in the range of 90 to 110 bp specifically for the use of real-time RT-PCR. Location of primers was preferentially closer to the 5’ end of the mRNA in order to distinguish artifacts from SAGE tags, and avoid known polymorphisms. Size of amplicons was confirmed by running the RT-PCR products on polyacrylamide gel as described above (data not shown). Fourty-eight primer pairs were designed and synthesized (Invitrogen) for the down-regulated mRNAs (p value < 0.001), and for other mRNAs of interest, including housekeeping genes. Table 2.1 shows a list of all primer pairs which generated single specific PCR products of the desired length. These primer pairs also amplified the target transcripts with similar efficiency over a 10000-fold range of input material.
<table>
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<tr>
<th>mRNA</th>
<th>5' Primer</th>
<th>Tm</th>
<th>GC</th>
<th>3' Primer</th>
<th>Tm</th>
<th>GC</th>
<th>Size</th>
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Table 2.1. Primer pairs designed for SAGE validation of libraries of IFNγ / LPS stimulated THP-1 cells, in the absence or presence of an 8 hour RAA (1 μM) treatment.

Full name of each mRNA is listed on Table 3.1 (in the Results section). *AS, tag-to-gene identities were assigned to antisense transcripts of genes. mRNAs for COX2 and IL6 which did not show up on SAGE data were included based on previous results in studies of Kastelic et al. (64). COX1 and IL-1α were included for comparison with COX2 and IL-1β mRNA expression. Housekeeping genes were included as well. Melting temperature (Tm), GC content (GC) of the primer, and also size of the amplicon are shown as well.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>5' Primer</th>
<th>Tm</th>
<th>GC</th>
<th>3' Primer</th>
<th>Tm</th>
<th>GC</th>
<th>Size</th>
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2.9 Real-Time RT-PCR

Real-time RT-PCR analysis was performed using the primers shown in Table 2.1. Real-time RT-PCR reactions were performed on an ABI PRISM 7900 Sequence Detection System (PE Applied Biosystems) using the SYBR Green I PCR Master Mix (Cat. # 4309155, PE Applied Biosystems) according to the manufacturer's instructions (PE Applied Biosystems, User Bulletin #2 applied to the SYBR-Green I core reagent protocol). The total reaction volume was scaled down proportionally to 15 μL per reaction: 7.5 μL of SYBR Green I PCR Master Mix, 1.25 μL of each 2 μM forward and reverse primers, 1.0 μL of 1:8-diluted template cDNA from the RT reaction as described in section 2.4, and 4.0 μL RNase-free water. Cycling was performed using the default condition of the ABI Prism 7900 SDS Software v2.0. Baseline default was CT = 3 to 15, and threshold default was 10 standard deviations above mean fluorescence generated during baseline cycles. Real-time PCR conditions were composed of 4 stages. First, an amplification step initiated the reaction by running the PCR plate at 50 °C for 2 min. Next, it was followed by an initial denaturation step at 95 °C for 10 min. Then, the denaturation / annealing / extension step carried through 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and at 72 °C for 30 s. Finally, the PCR reaction was terminated after incubation at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s, followed by incubation at 4 °C. Melting curve analysis on the dissociation plot (Fig. 2.1 B) showed a single melting peak, implying a single PCR product. If primer dimers or nonspecific fragments formed, peaks would also appear at lower temperatures. PCR reactions were performed in triplicate and included negative no-template controls to ensure reproducibility of the technique.
2.10 Relative Quantification in Real-Time RT-PCR

Relative changes in mRNA expression were calculated using the comparative $\Delta\Delta C_T$ (crossing point) method (User Bulletin #2, ABI PRISM 7700 Sequence Detection System). $C_T$ was determined automatically by the ABI Prism 7900 SDS 2.0 software as the second derivative maximum of the amplification curve. The $C_T$ value obtained from real-time RT-PCR is exponentially related to copy number. A PCR efficiency correction was determined for each assay by plotting standard curves of $C_T$ from eight serial dilutions in steps of two-fold for every primer pair against the log of total RNA (Fig. 2.1 C). Amplification efficiencies (E) were calculated from standard curves using the formula: $E = [10^{-1/slope}] - 1$. The amplification efficiency was assumed to be the same for all subsequent reactions, in spite of small fluctuations (1.0 ± 0.1).

The relative expression level of each mRNA was normalized to SNAPC2 (small nuclear RNA activating complex, polypeptide 2, 45kDa), the reference mRNA calibrator / housekeeping gene, and to the no-treatment control samples. SNAPC2 mRNA was insensitive to both THP-1 differentiation and RAA treatment (data are shown in Results section). The normalized relative expression level for each mRNA was calculated using the following formula:

$$\text{Normalized relative expression} = 2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T = \Delta C_T \text{ (treatment)} - \Delta C_T \text{ (control)}$, and $\Delta C_T$ is the $C_T$ of the target gene subtracted from the $C_T$ of the housekeeping gene. This equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator. For the $\Delta\Delta C_T$ method to be applicable, the PCR amplification
efficiency for the target mRNA should be approximately equal to that for the housekeeping gene. The amplification efficiencies for the target and housekeeping mRNAs were in proximity to 1.0 ± 0.1 in this case. Fold change ratio was calculated using the following formula:

$$\text{Ratio} = \frac{(1 + E_{\text{target}}) \Delta CT_{\text{target}} (\text{control} - \text{sample})}{(1 + E_{\text{ref}}) \Delta CT_{\text{ref}} (\text{control} - \text{sample})}$$
C.

Figure 2.1. Analyses of real-time RT-PCR results of RPL32 mRNA: (A) Amplification plot of serial dilutions; (B) Melting curve analysis – dissociation plot; and (C) Standard curve: log of total RNA versus CT.

(A) Eight serially diluted samples from RT reaction (cDNA, ranging from 1 to 128 dilutions) were detected using the primer pair specific for ribosomal protein L32 (RPL32) mRNA, as an example. The ABI SDS 2.0 software plotted fluorescence against cycle number, the fractional cycles (threshold cycle, C_T) at which the fluorescence signal becomes significantly different from the baseline signal.

(B) The dissociation plot of RPL32 mRNA was performed by plotting the first negative derivative of the fluorescence with respect to temperature against temperature. It shows a single melting maximum indicating specific amplification without forming primer dimers.

(C) The fractional cycles were plotted against the logarithm of the initial total RNA in samples. A linear relationship generates a correlation coefficient of r^2 > 0.99. The amplification efficiency, obtained from the slope of the standard curve, was (2.1 - 1) = 1.1 copies per cycle, according to the equation E = 10^{(-1 / \text{slope})} - 1. A negative amplification control was performed with a sample containing no cDNA. Quantitation of sample mRNA was achieved by extrapolation of fluorescence signals at the threshold cycle against the standard curves which represent the initial total RNA in samples for a defined fluorescence signal.
2.11 Absolute Quantification in Real-Time RT-PCR

The absolute quantification of any mRNA can be obtained from real-time RT-PCR if a standard curve of known quantity of cDNA is available. The target mRNA copy number of cDNA samples was generated from the calibration curves of a real-time PCR amplicon, based on a modified method of Ovstebo et al. (103), which used an mRNA calibrator as a reference. Two PCR calibrators, SNAPC2 and CCL5 (Chemokine CC motif ligand 5), were chosen for this project. The average melting temperature for the primer pair of SNAPC2 is 56 °C and CCL5 is 60 °C, and their amplicon sizes are 91 and 113 bp, respectively. Primer pairs of all genes in this project had melting temperatures and amplicon sizes within the range of these two calibrators. Testing both the highest and the lowest melting temperatures, and amplicon sizes would give confidence in the accuracy and precision of absolute quantification calculation.

mRNA was isolated and reverse-transcribed as described in section 2.4. Specific PCR primer pairs of each calibrator (SNAPC2 and CCL5, see Table 2.1) were used to amplify the calibrators in large-scale. PCR products were separated using 8 % PAGE, as described in section 2.5, to obtain the expected size amplicons. PCR amplicons were purified using phenol-chloroform extraction and ethanol precipitation, as described in section 2.6. The amplicon concentration was determined using a UV spectrophotometer. The absorbance of the purified PCR calibrators at 260 / 280 nm revealed the concentration and purity of PCR products, as described in section 2.4. The amplicon copy number was calculated from the PCR-amplified cDNA concentration, as well as taking into account the size of each specific
PCR calibrator, the molecular weight of the nucleotide base pair (g / mol), and Avogadro’s number \(6.022 \times 10^{23}\). The formula for calculating the amplicon copy number is as follows:

\[
\text{Copies of molecule per 1 \(\mu\)L cDNA volume} = \text{concentration of cDNA} \times \text{Avogadro’s number} / \text{amplicon length} / \text{molecular weight per base pair}.
\]

A series of eight ten-fold dilutions (Fig. 2.1 A) from each calibrator (in the range of \(10^2\) to \(10^9\) cDNA molecules) was examined using real-time RT-PCR to calculate a standard curve. CT values were plotted against the log of the initial template concentrations. All transcripts showed optimal PCR efficiencies, in the range of 1.0 to \(1 \times 10^5\) pg of total RNA input with high linearity. The copy number was calculated from a linear regression of the data. The reproducibility of the calibration curves were examined by calculating mean and range values for each dilution.

### 2.12 Antisense Strand-Specific Real-Time RT-PCR

Some SAGE tags did not match to any EST or predicted exons of known mRNAs. Instead, DiscoverySpace software and other SAGE database (described in section 2.7) matched these tags to the antisense strand of known genes. Some of these SAGE tags matched to single mRNA, whereas most of them matched to multiple genes. In order to hybridize with and thus detect an antisense strand-specific mRNA, the probe must consist of the sense sequence. Real-time RT-PCR was used to confirm the prospective antisense SAGE tags. Briefly, 3 sets of antisense strand specific primers were designed for three selected mRNAs: CCL3, CCL4, and CCL8. The antisense SAGE tags of these 3 mRNAs were in the 3’ UTR of the coding gene. These primer pairs all had a reverse (3’-) primer that primed beyond the 3’ UTR of
coding mRNAs determined from genomic sequence, together with a forward (5'-) primer within the 3' UTR. Then, the antisense SAGE tag would locate within the PCR products of these primer pairs. Instead of using oligo-dT primers, the first-strand cDNAs were reverse-transcribed using the antisense transcript-specific 5'- primer that located within the 3' UTR. Thus, the first strand cDNA would only include specific antisense transcripts. The cDNA sample was then amplified with the antisense primer pairs (both 5'- and 3'- primers under the same final concentration) using real-time RT-PCR, as described in section 2.9.
<table>
<thead>
<tr>
<th>#</th>
<th>mRNA</th>
<th>*5' Position of Primer</th>
<th>Primer Sequence (5' to 3')</th>
<th>Primer Orientation</th>
<th>Size of Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCL4</td>
<td>563</td>
<td>GTTTAGCCAAAGGATAAGTGC</td>
<td>Forward</td>
<td>111</td>
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<tr>
<td>2</td>
<td>CCL4</td>
<td>673</td>
<td>GTTTTATTTGAAAACACAGAGATAAC</td>
<td>Reverse</td>
<td>103</td>
</tr>
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<td>3</td>
<td>CCL4</td>
<td>643</td>
<td>CATTTGATCTGTGTTTTTCATAAAAAC</td>
<td>Forward</td>
<td></td>
</tr>
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<td>5</td>
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<td>631</td>
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<td>108</td>
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<tr>
<td>6</td>
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<td>738</td>
<td>GATCACAGGCCCTGAAACAAAAG</td>
<td>Reverse</td>
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</tr>
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<td>7</td>
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<td>717</td>
<td>CTTTTGTTCAGGCTGTGATC</td>
<td>Forward</td>
<td>107</td>
</tr>
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<td>8</td>
<td>CCL4</td>
<td>823</td>
<td>CAGTGATTGTTGATTTGCCAGGAA</td>
<td>Reverse</td>
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<tr>
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<td>1293</td>
<td>CTCACCTCCAGCTAAATGTA</td>
<td>Reverse</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Primer pairs designed for validating SAGE tags that encode antisense mRNAs.

* 5' position of each primer is based on the mRNA sense sequence accessed from the GenBank. Accession number of CCL4 mRNA is NM_002984; CCL3 mRNA, NM_002983; and CCL8 mRNA, NM_005623. Size of amplicon was determined by forward (5') and reverse (3') primers of each mRNA. Some primers were located within the 3' UTR, whereas others were located downstream of the 3' UTR. Sequences of the latter were obtained from the DNA sequence of specific chromosome where the gene was located. Accession number of genomic sequence corresponding to CCL4 mRNA is AC003976.1; CCL3 mRNA, AC069363.10; and CCL8 mRNA, X99886.1.
2.13 Determination of Optimal Actinomycin D Concentration by FACS

In order to study mRNA half-life, the synthesis of newly transcribed RNA needs to be blocked. The optimal concentration of ActD (cell culture grade, Cat. # A9415, Sigma), a DNA-dependent RNA polymerase inhibitor for all three types of RNA polymerases (I, II, III), was chosen for THP-1 cells such that mRNA transcription would be virtually inhibited while maintaining survival of most cells. In brief, the newly synthesized mRNAs in THP-1 cells would not incorporate added 5-bromouridine 5'-triphosphate (BrUTP) (Cat. # B7166, Sigma) when ActD blocked transcription (35). Cells (1 x 10^6 cells / ml) were plated in a 6-well plate, and induced to differentiate as described in section 2.1. ActD at various final concentrations [50, 20, 5, 1, and 0.01 μg / mL, prepared in dimethyl sulfoxide (DMSO, sterile-filtered, Cat. # D2650, Sigma)] was added to the cells. Cells were washed with Wash Buffer [Dulbecco’s PBS (Gibco) with 2 v / v % FBS (Gibco)] at either 15 or 240 min after treatment with ActD. The 6-well plate was centrifuged at 1500 RPM at 4 °C for 5 min, and supernatant media was discarded without disturbing the cells in each wash. BrUTP was transfected into the cells using FuGENE Lipofection (Roche), following the manufacturer’s instructions. Briefly, FuGENE was 10 % diluted with serum-free RPMI 1640. After a 5 min incubation at room temperature, BrUTP was added to the diluted FuGENE solution to a final concentration of 0.7 mM, and incubated for 15 min at room temperature. Subsequently, 0.6 mL BrUTP / FuGENE mixture was added to each well. After a 15 min incubation at 37 °C in a 95 % humidified incubator with 5 % CO₂, BrUTP-transfected cells were washed with Wash Buffer, and detached with 0.2 % EDTA (Sigma) in PBS (Gibco) at 37 °C. The 6-well plate was centrifuged at 1500 RPM at 4 °C for 5 min, and drained after each wash. Then cells were fixed with 1 % paraformaldehyde (Sigma) prepared in PBS at pH 7.5, and
permeabilized with 100% cold methanol. Cells were then incubated with Permeate Buffer
[(Dulbecco's PBS with 4 v/v % FBS, and 0.2 v/v % Triton X-100 (Sigma)] containing an
anti-BrdU primary antibody conjugated with Alexa Fluor 488 (mouse IgG, monoclonal PRB-
1, Cat. # A21303, Molecular Probes), diluted to a final concentration of 6 μg/mL. After
immuno-staining for an hour at room temperature, fixed cells were washed two times each
with Permeate Buffer first and then with Wash Buffer at room temperature. The
fluorescence was measured using a Fluorescence-Activated Cell Sorter (Beckman Coulter
Epics Elite ESP) at 488 nm excitation and 525 nm emission. The percentage of transcription
inhibition was calculated using the formula (fluorescence intensity = I):

\[ \%_{\text{Inhibition}} = \left( 1 - \frac{I(\text{Sample}) - I(\text{No BrUTP})}{I(\text{No ActD})} \right) \times 100 \]

2.14 In vitro Half-Life Determination

In order to study mRNA half-life, THP-1 cell cultures were maintained as described in
section 2.1. Various treatments with either ActD (5 μg/mL), RAA (1 μM), both ActD (5 μg
/mL) and RAA (1 μM), or control vehicle alone (DMSO, final concentration = 0.1 %, and
methanol, final concentration = 0.1 %), were administered at three stages of differentiation:
monocytes (t = -8 h), and 2 and 16 h post-IFNγ / LPS stimulated THP-1 cells. Total RNA
was sequentially isolated at times 0, 15, 45, 75, 135, and 240 min after treatment. Half-life
of ten representative mRNAs: CCL4, NFKBIA, IL6, IL-1β, TNF-α, IL10RA, COX2,
TMSB4X, KYNU, and CCL5 (as shown in Table 3.3 in Results section), was examined
using real time RT-PCR. mRNA decay was determined by plotting the copy number per μg
RNA against the time of ActD treatment. The slope and Y-intercept was determined by
Prism 3.0 statistical software (GraphPad Software Inc., San Diego, CA) using linear regression. mRNA half-life was calculated using the formula:

\[
\text{mRNA half-life (t_{1/2})} = \text{slope}^{-1} \times \ln 2
\]

2.15 Statistical Analyses

To assess variations in gene expression measurements using real-time RT-PCR, statistical analysis was performed with Prism 3.0 statistical software. Linear regression was applied to correlate two variables. Pearson correlation coefficients (r) were used for statistical comparisons of the differences seen in expression levels between the samples. Statistics were assessed using a Student’s t-test, assuming double-sided independent variance and with p < 0.05 considered to be significant. All values are given as mean ± standard error of the mean (SEM).
3.0 Results

3.1 SAGE Data Mining

Two SAGE libraries from IFNγ / LPS-induced THP-1 cells (as described in 2.1, in the absence of βME) treated with RAA (1 μM) or methanol for 8 hours were constructed in order to investigate the effect of RAA on the entire transcriptome. A total of 90,905 tags from both SAGE libraries [untreated (47,210 tags) and RAA-treated (43,695 tags)] were sequenced at GSC. DiscoverySpace software identified approximately 21,600 valid tags from both libraries. A total of 17,608 unique tags were determined for both libraries. The average frequency per tag is about 2. Only 218 tags of the total tags sequenced were down-regulated by more than 3-fold. Of the total tags, 182 tags which were down-regulated by RAA, and 243 tags which were up-regulated by RAA, had high transcript expression (tag number > 15). At a 95.0 % confidence level as shown in Figure 3.1, 97.77 % of the tags remained unchanged, whereas only 0.96 % (169 tags) was down-regulated, and 1.27 % (223 tags) was up-regulated by RAA treatment. Even though 97.77 % of the tags remained unchanged at the confidence level of 95.0 %, changes in low abundance mRNAs such as COX2 mRNA, would not be detected by SAGE libraries having only ∼45,000 tags sequenced. At a confidence level of 99.9 %, 48 tags (0.27 %) were down-regulated and 29 tags (0.16 %) were up-regulated by RAA treatment (Fig. 3.1).
Figure 3.1. Distribution of tags from two SAGE libraries in the absence or presence of an 8 hour RAA (1 μM) treatment in IFNγ / LPS stimulated THP-1 cells.

DiscoverySpace software generated a SAGE tag analysis to visualize the change in mRNA expression level upon the RAA treatment. Number of tags in the RAA-treated library were plotted against the number of tags in the RAA-untreated library. The levels of confidence were shown in the form of a tunnel. Tags above the corresponding level of confidence from the diagonal tunnel were significantly up-regulated, whereas tags below the diagonal tunnel were significantly down-regulated. RAA down-regulated tags at a confidence level of 99.9% were labeled red.
mRNAs down-regulated by RAA were the primary focus of this study, as a continuation of the work of Kastelic et al. (64) but at a transcriptome-wide level. Table 3.1 shows the set of 48 SAGE tags, which were down-regulated upon RAA treatment in IFNγ / LPS-induced THP-1 cells. The most down-regulated tag identified encoded CCL4 mRNA, which had a reduction over a thousand fold (Table 3.1). Fold changes were normalized from raw data of both libraries. However, CCL4 mRNA was also the third most down-regulated tag identified, with a 230 fold decrease. Therefore, the frequency of these two tags were summed and compared between libraries in order to calculate the actual fold decrease. For instance, CCL4 mRNA would have 492 fold decrease after taking into account the presence of two tags mapping to a single mRNA. For reference, the number of SAGE tag which encodes the housekeeping ribosomal protein L32 (RPL32) is 65 in the RAA-untreated library, comparing to 108 in the RAA treated library. Upon RAA treatment, it appears that many mRNAs encoding inflammatory cytokines, such as the chemokine CC ligand and interleukin families, were significantly down-regulated, as observed in Table 3.1.
<table>
<thead>
<tr>
<th>#</th>
<th>Sequence</th>
<th>Tag counts</th>
<th>Fold Decrease</th>
<th>p-value</th>
<th>Potential Identities Tag to Gene Association</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>untreated</td>
<td>treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>TGTTTTTCATA</td>
<td>1133</td>
<td>1</td>
<td>1048.6</td>
<td>CCL4 - chemokine (C-C motif) ligand 4</td>
</tr>
<tr>
<td>2</td>
<td>GCCCAAAAGC</td>
<td>606</td>
<td>3</td>
<td>187.0</td>
<td>CCL3 - chemokine (C-C motif) ligand 3</td>
</tr>
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<td>463</td>
<td>2</td>
<td>214.3</td>
<td>CCL4 - chemokine (C-C motif) ligand 4</td>
</tr>
<tr>
<td>4</td>
<td>TGGAAAGCCT</td>
<td>109</td>
<td>0</td>
<td>100.9</td>
<td>IL8 - interleukin 8</td>
</tr>
<tr>
<td>5</td>
<td>AAAATCGGC</td>
<td>358</td>
<td>124</td>
<td>2.7</td>
<td>CCL5 - chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
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<td>GATCATCAAAG</td>
<td>87</td>
<td>2</td>
<td>40.3</td>
<td>CCL8 - chemokine (C-C motif) ligand 8</td>
</tr>
<tr>
<td>7</td>
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<td>66.6</td>
<td>IL1B - interleukin 1, beta</td>
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<td>40</td>
<td>4.1</td>
<td>CXCL10 - chemokine (C-X-C motif) ligand 10</td>
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<td>50.9</td>
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<td>49.1</td>
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</tr>
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<td>1</td>
<td>41.6</td>
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</tr>
<tr>
<td>13</td>
<td>TGATGCTGAC</td>
<td>45</td>
<td>1</td>
<td>41.6</td>
<td>IL23 - interleukin 23, alpha subunit p19</td>
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<td>14</td>
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<td>473</td>
<td>1.5</td>
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<td>3</td>
<td>14.8</td>
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<td>17</td>
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<td>11</td>
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<td>CHORDC1 - Cys and His-rich domain-containing, zinc binding protein 1</td>
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<td>8.9</td>
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<td>19</td>
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<td>8</td>
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<td></td>
<td></td>
<td></td>
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<td>35</td>
<td>2.6</td>
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<td></td>
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<td>0</td>
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<td>2.15E-06</td>
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<td>9</td>
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<tr>
<td>41</td>
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<td>3.81E-04</td>
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<td>7</td>
<td>3.8</td>
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<td>44</td>
<td>GTGACCACGG</td>
<td></td>
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</tr>
</tbody>
</table>

* KCNS1 - potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
* NFKB2 - nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49 / p100)
* CCL8 - chemokine (C-C motif) ligand 8
* GPR73 - G protein-coupled receptor 73
### Table 3.1. Tag-to-gene mapping of two SAGE libraries in the absence or presence of an 8 hour RAA (1 μM) treatment in IFNγ / LPS stimulated THP-1 cells.

SAGE tag sequences of 99.9% confidence were ranked in ascending p value. All tag counts are raw data. Fold decrease was normalized to both libraries. Fold decrease was calculated from the ratio of the frequency of each SAGE tag in the untreated library to the library with RAA treatment. Fold decrease was calculated by assuming the number of tags in RAA treated library to be 1 if the tag count was 0. Exponent of ten is expressed using the notation of E. Some SAGE tags had more than one potential gene identities assigned. * SAGE tag mapped to the antisense strand. The average of fold decrease in multiple tags mapping to an mRNA was calculated: CCL3 mRNA, 193 fold decrease; CCL4 mRNA, 492 fold decrease; CCL5 mRNA, 2.65 fold decrease; NFKBIA mRNA, 5.92 fold decrease; TNFSF10 mRNA, 8.13 fold decrease; and SOD2 mRNA, 3.50 fold decrease.
Most of the tag-to-gene identities, determined using the DiscoverySpace software, BLAST of the NCBI database, SAGEmap, and SAGE genie, were consistent. Some of the SAGE tags led to multiple identities which also included expression from antisense mRNA. A number of the SAGE tags were assigned to multiple identities because none of the tag-to-gene identities gave a perfect match to any known genes or EST, indicating a possibility of discovering unknown genes. In general, if SAGE tags identified were not the first or second tag from the 3' end of mRNA, the tag-to-gene identification would be more questionable, unless an internal poly-A sequence was found. For example, tags encoding CCL5 mRNA appeared twice in Table 3.1: one tag was located closest to the 3' UTR and the other one was the third tag from the 3' UTR. The internal poly-A stretch on CCL5 mRNA located next to the third tag might be primed by the oligo-dT primers when preparing the SAGE libraries. Of the 48 tags, 9 tags encoded antisense transcripts, 10 tags had multiple mRNA identities, and 6 of the mRNAs had more than one tag.

The SAGE libraries were further investigated for the occurrence of unmatched tags and SK box-containing genes. Even though DiscoverySpace software attempted to map each SAGE tag to its corresponding gene, there were tags that did not map to any mRNA in reality, and they were categorized as unmatched or unassigned tags. Tags were categorized into up-regulated and down-regulated groups based on the tag count from both libraries. When the ratio of tag counts from the RAA-treated library to the untreated one was less than 1.00, the tag was classified as down-regulated, and vice versa. Then, tags in each category (up-, down-regulated, and unchanged) were arranged in descending order according to the tag count found in SAGE libraries. Most of the tags in the unchanged category were only
expressed once. Of the total unique tags, 800 SAGE tags (first 400 down-regulated, and first 400 up-regulated) were analyzed through accessing the Ensembl Human Transcript database, the Human UniGene of NCBI GenBank database, and NCBI Human Reference Sequences (RefSeq) database in order to obtain a tag-to-gene map. Among the down-regulated tags, 12.3 % (49 out of 400 tags) were unmatched to any existing genes or EST. The up-regulated tags had 9.3 % (37 out of 400 tags) unmatched tags. Then, gene identities of tags which have been assigned were queried on the ARED online database to determine the presence of SK boxes. The down-regulated tags had 8.5 % (34 out of 400 tags) that contained at least one SK box, but the un-regulated tags had only 1.5 % (6 out of 400 tags). Of the 48 down-regulated tags (at a confidence level of 99.9 %), 13 tags (27.1 %) had gene identities that contained SK boxes.

3.2 Identification of a Housekeeping Gene – SNAPC2 mRNA

To determine the relative quantification of real-time RT-PCR results, a stably expressed reference mRNA, usually a “housekeeping” gene, is required for normalization. The mRNA levels of four common housekeeping mRNAs: β-actin (ACTB), β-2-microglobulin (B2M), GAPDH, and ribosomal protein L32 (RPL32), were examined during THP-1 differentiation and RAA treatment in order to identify an mRNA that does not vary under the conditions applied. However, all these 4 mRNAs were sensitive to the differentiation protocol of THP-1 cells based on real-time RT-PCR results, as shown in Figure 3.2 C. SNAPC2 mRNA was an mRNA that shared its tag with USP18 mRNA. Unexpectedly, the mRNA expression level of SNAPC2 did not change during the course of THP-1 differentiation from monocyte to macrophage (Fig 3.2 B), and was insensitive to RAA (1 μM) (Fig 3.2 A). Although
SNAPC2 mRNA has a high $C_T$ value, that is, a low expression level, the variation was low signifying a constant expression. SNAPC2 mRNA was used as the calibrator and included to normalize the mRNA expression at each stage of differentiation in THP-1 cells.
B.

![Graph showing CT values over time after LPS addition](image)

- Time after LPS addition (h)
- CT values range from 20 to 40
Figure 3.2. mRNA expression level of SNAPC2 and other housekeeping genes in IFNγ / LPS stimulated THP-1 cells (t = 24 h) ± RAA (1 μM) treatment.

(A) The Ct values for SNAPC2 and other housekeeping mRNAs in IFNγ / LPS stimulated THP-1 cells (t = 24 h) ± RAA (1 μM) treatment were plotted. (B) The Ct values for SNAPC2 mRNA during THP-1 differentiation upon IFNγ (t = -3 h) and LPS (t = 0 h) treatment were plotted as a function of time after LPS addition. All real-time RT-PCR data shown are in raw Ct values. (C) Relative mRNA expression levels for β-Actin (ACTB), β-2-microglobulin (B2M), GAPDH, and RPL32 mRNAs were plotted as a function of time during THP-1 differentiation upon IFNγ / LPS treatment. The ΔΔCt method was used to calculate the real-time RT-PCR fold change using SNAPC2 mRNA for normalization. Some error bars are too close to be resolved.
3.3 SAGE Data Validation

Due to multiple gene-to-tag mapping and low sampling size of the libraries that were sequenced, validation of the SAGE libraries was required. RAA-down-regulated mRNAs corresponding to a confidence level of 99.9% (48 tags), which mapped to 53 mRNAs, were verified using real-time RT-PCR. Total RNA was isolated from IFNγ / LPS stimulated THP-1 cells (t = 24 h) in the absence or presence of an 8 hour RAA (1 μM) treatment, under the same conditions as the SAGE libraries were constructed. 44 mRNAs were examined using real-time RT-PCR, excluding antisense mRNAs. In addition, COX1, COX2, IL-1α, and IL6 mRNAs were included as well, even though these 4 mRNAs were not detected in the SAGE libraries. mRNAs for COX2 and IL6 were included based on previous results from Kastelic et al. (64). COX1 and IL-1α were included for comparison with COX2 and IL-1β mRNA expression.

From the real-time RT-PCR results, 37 mRNAs were still found to be sensitive to RAA. Table 3.2 is a summary of the fold decrease in real-time RT-PCR and SAGE results of these 37 mRNAs. mRNAs were ranked in descending order of the fold decrease in mRNA expression upon RAA treatment. Although the fold decreases in mRNA expression between real-time RT-PCR and SAGE were not exactly identical, they were of the same magnitude with similar fold changes. Real-time RT-PCR is more sensitive to low mRNA levels than SAGE libraries with low number of tags sampled. In general, CT values greater than 26 (signifying low expression) from real-time RT-PCR were not detected in the ~45,000-tag SAGE libraries. Using a cut off at 1.5-fold decrease in real-time RT-PCR results, 26 mRNAs were found to be sensitive to RAA (Table 3.2). The majority of these mRNAs (73 %)
examined contained AREs, and 69% encoded cytokines, such as CC chemokine ligand and interleukin families.
<table>
<thead>
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<th>mRNA</th>
<th>Description</th>
<th>Real-time RT-PCR</th>
<th>SAGE</th>
<th># of SK boxes</th>
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<td>0.8</td>
<td>*</td>
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</table>

Table 3.2. A comparison of number of SK boxes found in 48 RAA down-regulated mRNAs, and the fold decrease obtained with SAGE and real-time RT-PCR in IFNγ / LPS stimulated THP-1 cells ± RAA (1 μM) treatment.
Total RNA isolated from IFNγ / LPS stimulated THP-1 cells (t = 24 h) in the absence or presence of an 8 hour RAA (1μM) treatment was under the same condition as the SAGE libraries were constructed. mRNAs were ranked based on the descending fold decrease in mRNA expression upon RAA treatment from real-time RT-PCR results. The ΔΔC_{T} method was used to calculate the real-time RT-PCR fold change using SNAPC2 mRNA for normalization. Fold decrease was calculated by assuming the number of tags in the RAA-treated SAGE library to be 1 if the tag count was 0. * mRNAs for COX2 and IL6 which did not show up on SAGE data were included based on previous results (64). COX1 and IL-1α were included for comparison with COX2 and IL-1β mRNA expression.
As shown in Table 3.2, the number of SK boxes found on the 3' UTR of each mRNA was found to vary greatly from 0 to 17 copies. Figure 3.3A shows a correlation between the total number of SK boxes and RAA sensitivity. As the regression analysis for the fold decrease in mRNA expression levels and the number of SK boxes was plotted on Figure 3.3, the coefficient of determination, $r^2 = 0.13$, indicated that only 13% of mRNAs down-regulated by RAA would be explained by these two variables. The closer the absolute $r^2$ approaches to 0, the less the correlation between the two variables. Thus, the total number of SK boxes does not simply correlate with the extent of mRNA down-regulation in cells treated with RAA. For example, IL-1β mRNA which contained 6 SK boxes had a 53-fold decrease, compared to COX2 mRNA which contained 22 SK boxes but only had a 7-fold decrease upon RAA treatment. Interestingly, mRNAs with the most fold decrease contained 5-6 SK boxes in tandem. Indeed, a relatively stronger linear relationship between the fold decrease in mRNA expression and number of SK boxes in tandem with a correlation coefficient of $r^2 = 0.41$ was shown in Figure 3.3B when we categorized mRNAs based on their number of overlapping SK boxes according to the ARED classification. The more overlapping SK boxes an mRNA has, the stronger the prediction of the RAA-mediated down-regulation would be on that mRNA. The organization, rather than the total number, of SK boxes shows a stronger prediction toward RAA sensitivity.
A.

![Graph showing the relationship between the number of SK boxes and fold decrease based on real-time RT-PCR results. The equation of the regression line is $y = 1.205x + 3.8127$ with $r^2 = 0.132$.](image)
B.

Figure 3.3. Fold decrease in mRNA expression (±1 µM RAA) based on real-time RT-PCR results against the number of SK boxes in 37 mRNAs, listed in Table 3.2.

Data was taken from Table 3.2. The coefficient of determination, $r^2$, is the percentage of variability (as measured by the sum of the squared residuals) that is explained by the regression line.

(A) The plot of fold decrease in mRNA expression versus number of SK boxes shows a weak linear relationship with a correlation coefficient of $r^2 = 0.13$.

(B) mRNAs were categorized based on their number of overlapping SK boxes according to the ARED classification: ARED Group I, II, III, IV, and V contain 5, 4, 3, 2, and 1 overlapping SK boxes, respectively. A relatively stronger linear relationship with a correlation coefficient of $r^2 = 0.41$ was found between the fold decrease in mRNA expression and number of SK boxes in tandem.
3.4 Antisense Strand Expression Determination

Of the 48 down-regulated tags (confidence level of 99.9 %), 9 tags (19 %) were ascribed to antisense mRNA. Of these 9 tags, only 5 tags mapped to antisense mRNA alone and did not match to other known mRNAs or ESTs. To determine potential mRNA expression from the antisense strands, three mRNAs: CCL3, CCL4, and CCL8 were chosen to be examined. These three antisense mRNAs were chosen because their sense mRNAs showed a great sensitivity toward RAA treatment (referred to Table 3.1) in SAGE data, and also their antisense transcript-encoding SAGE tags were the first tag from the 3' UTR.

As shown in Figure 3.4, antisense mRNA expression was not down-regulated by RAA, but rather was up-regulated slightly. The average fold decrease based on real-time RT-PCR for CCL3 antisense mRNA is 0.93, CCL4 antisense mRNA is 0.54, and CCL8 antisense mRNA is 0.67. However, based on the SAGE data, CCL3 and CCL8 antisense mRNAs had 16-fold decrease, and CCL4 antisense mRNA which matched to two tags has over 50-fold decrease. The results from real-time RT-PCR did not match with the SAGE data at all. All of these antisense mRNA had very low expression, and their average $C_T$ was over 30. $C_T$ for CCL3 antisense mRNA is 32.3, CCL4 antisense mRNA is 33.9, and CCL8 antisense mRNA is 32.0. SAGE data indicated that these antisense transcripts had relatively high expression (tag count $\geq 15$), but real-time RT-PCR results did not agree with the fold decrease in SAGE data at all.
Figure 3.4. Comparison of the fold decrease in antisense mRNA expression upon RAA treatment based on real-time RT-PCR.

Total RNA isolated from IFNγ / LPS stimulated THP-1 cells (t = 24 h) in the absence or presence of an 8 hour RAA (1μM) treatment was reverse-transcribed using the antisense strand-specific 5’ primer located within the 3’ UTR instead of using oligo-dT. Each antisense mRNA was primed using a pair of antisense strand-specific primers as indicated in brackets (see Table 2.2 in Materials and Methods section). The numbers in brackets after the mRNA refer to the primer pairs used as per Table 2.2. The ΔΔC_T method was used to calculate the real-time RT-PCR fold change using SNAPC2 mRNA for normalization.
3.5 Time Course Study of Gene Expression

The steady-state levels of housekeeping and RAA-sensitive mRNAs were investigated under a time course study of mRNA expression during THP-1 differentiation. These mRNAs included β-actin, β-2-microglobulin, GAPDH, ribosomal protein L32, BCL2A1, CCL2, CCL3, CCL4, CXCL10, COX1, COX2, CYP3, IL-1α, IL-1β, IL6, IL8, IL10RA, NFKBIA, RNF8, SNAPC2, TNF-α, TNFSF10, and USP18. mRNA expression in THP-1 cells was plotted as a function of time after LPS stimulation. The three mRNA expression graphs shown in Figure 3.5 were arranged such that selected mRNAs were grouped according to similar expression patterns. Again, as stated in section 3.2, SNAPC2 was chosen as the housekeeping mRNA, which was insensitive to both THP-1 differentiation protocol and RAA treatment (Fig. 3.2), for real-time RT-PCR results normalization.

As can be seen on Figure 3.2 C, β-actin, GAPDH, and ribosomal protein L32 mRNAs all had an immediate increase in expression level after IFNγ / LPS stimulation but slowly came down at later hours. β-2-microglobulin mRNA gradually peaked at 9 h, and remained high. mRNA expression of these four common housekeeping mRNAs was affected by the THP-1 differentiation protocol.

Figure 3.5 A shows a representative mRNA expression pattern of the early-intermediate response gene family during the course of THP-1 differentiation. BCL2A1, COX1, COX2, IL-1α, IL-1β, IL8, NFKBIA, and TNF-α mRNAs all shared a similar pattern of mRNA expression upon IFNγ / LPS stimulation. As shown in Figure 3.5 A, the mRNA steady-state level of these RAA-sensitive mRNAs all increased relative to expression in THP-1
monocytes before the IFNγ / LPS treatment, except COX1 mRNA. These mRNAs showed a peak of expression at about 2 h post IFNγ / LPS stimulation, in an early-intermediate response. The mRNA steady-state level of all these mRNAs rose again at later hours during THP-1 differentiation, except TNF-α mRNA. Among these mRNAs, only TNF-α, NFKBIA, and COX2 mRNAs had a dramatically immediate reduction in mRNA expression after the expression peak at 2 h.

Figure 3.5 B shows another pattern of mRNA expression. Instead of an immediate response, CXCL10, IL10RA, TNFSF10, and USP18 mRNAs rose gradually upon IFNγ / LPS stimulation. Their mRNA expression peaked at about 8 h, and then decreased. TNFSF10 and USP18 mRNAs had a bump in their mRNA expression at 16 h, whereas CXCL10 mRNA steady-state level reduced sharply after peaking at 8 h. IL10RA mRNA expression showed a plateau after a peak at 8 h, indicating that it reached its specific steady-state level. Similarly, expression of CCL2, CCL3, CCL4, and IL6 mRNAs, as shown in Figure 3.5 C, all had a slower response toward IFNγ / LPS stimulation such that they did not reach their maximal expression until later hours (> 16 h). Both IL6 and CCL3 mRNAs continued their rise in expression after 16 h. The expression level of both CCL2 and CCL4 mRNAs peaked at 16 h, and then decreased. Even though the specific expression pattern of each mRNA did not match exactly, these selected mRNAs were grouped according to the specific time after stimulation that gave them their signature shape.
B.

![Graph showing relative mRNA expression normalized to SNAPC2 over time after LPS addition (h). The graph includes lines for CXCL10, IL10RA, TNFSF10, and USP18.](image)
Fold change in mRNA expression in THP-1 cells was plotted as a function of time after LPS addition. THP-1 cells was treated with IFNγ at -3 h, and then stimulated with LPS at 0 h. Total RNA was isolated from THP-1 cells at t = -3, 0, 1, 2, 4, 8, 12, 16, and 24 h. Fold changes in real-time RT-PCR results were based on normalization to SNACP2 using the ΔΔCₗ method. Error bars are too close to be resolved. mRNAs with similar patterns were grouped as shown in Figure 3.5 (A) to (C).
3.6 mRNA Half-Life

3.6.1 Use of FACS Analysis to Determine the Optimal Actinomycin D Concentration for THP-1 cells

In order to study mRNA half-life, mRNA transcription of THP-1 cells was inhibited with ActD, a RNA polymerase inhibitor. However, determining an optimal ActD concentration for THP-1 cells was important in order to inhibit mRNA transcription maximally while maintaining survival of most cells. The transcription rate as a function of ActD concentration was determined using FACS, which measures the fluorescence of newly transcribed BrUTP-incorporated mRNAs, labeled with an anti-BrUTP conjugated fluorescent antibody.

Results of FACS analysis on transcription inhibition in response to various concentrations of ActD (t = 15 min) in THP-1 cells stimulated with IFNγ / LPS (t = 16 h) are shown in Figure 3.6. It was found that FACS results from various stages of THP-1 differentiation (t = -8, 2, and 16 h) and various lengths of ActD incubation (t = 15 and 240 min) were similar. All samples were treated under the same condition except the unstained sample (no BrUTP added), and the no-treatment control sample (no ActD added). All fluorescence measurements were made on a four-decade log scale. A total of 10,000 events were collected for each sample. After adjustment of the background signal with the mean fluorescence of the unstained sample, the percentage of transcriptional inhibition, listed on Figure 3.6 B, was calculated using the mean fluorescence of each sample against the intensity of the sample without ActD inhibition. Shifting to stronger fluorescence intensity as the ActD concentration decreased indicated that mRNA transcription increased.
At 20 and 50 μg / mL ActD with a 15 min incubation, the transcription inhibition was 99 %, but the majority of the THP-1 cells were observed to have undergone apoptotic morphological changes: i.e. cells were detached, had irregular shapes, and nuclear bodies had formed. As the concentration of ActD was reduced to 5 and 1 μg / mL, fewer detached cells with abnormal morphology were observed while high mRNA transcription inhibition was maintained (98 % and 97 %, respectively). Transcription inhibition decreased slightly when THP-1 cells were incubated with 1 μg / mL ActD over 4 hours, but this was not observed at 5 μg / mL ActD. At 0.01 μg / mL ActD, mRNA transcription was only inhibited by 76 %. Thus, a concentration of 5 μg / mL ActD was chosen for the mRNA half-life study in order to achieve maximal transcription inhibition, yet minimizing cell death.
Figure 3.6. Inhibition of mRNA transcription in response to various concentrations of ActD in 16 h post-IFNγ/LPS stimulated THP-1 cells.

(A) Cell count was plotted as a function of log fluorescence intensity using FACS analysis. Because FACS results from various stages of THP-1 differentiation and various length of ActD incubation were similar, values shown here were an example from 16 h post-IFNγ/LPS treated THP-1 cells incubated with various concentration of ActD for 15 min. Percentage of inhibition is shown in (B).
3.6.2 Classification of mRNAs for mRNA Half-Life

Although it was found that the total number of SK boxes did not correlate with an mRNA’s sensitivity to RAA, the arrangement and organization of SK boxes was another important aspect to be investigated. Of the 37 RAA-sensitive mRNAs (shown in Table 3.2), 10 representative mRNAs (shown in Table 3.3) were chosen based on their ARE class, in order to further correlate the effect of RAA on mRNA half-life with SK box organization. These ten mRNAs included mRNAs that encoded both cytokines and other proteins, and mRNAs of all three classes of ARE (Table 1.1) based on the classification system of Chen and Shyu (29) and of the five ARED clustering groups (Table 1.2). The mRNAs were listed in descending order of their ARE classification based on Chen and Shyu. Although mRNAs lacking SK box (TMSB4X, KYNU, and CCL5) were down-regulated by RAA, their fold decreases as measured by real-time RT-PCR were generally less when compared to SAGE data.
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<th>Description</th>
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<th>Class of ARE (I, II, III or N=none)</th>
<th># of SK boxes in 3'UTR</th>
<th>ARED clustering group</th>
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<td>-</td>
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<tr>
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<td>N</td>
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<td>N</td>
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Table 3.3. ARE classifications of ten mRNAs that were examined for the effect of RAA on mRNA half-life in THP-1 cells (t = 24 h).

The ΔΔC_T method was used to calculate the fold decrease in real-time RT-PCR results (1 μM RAA, 8 h treatment) using the SNAPC2 mRNA calibrator.
3.6.3 mRNA Half-Life Determination

mRNA half-life was examined at three stages of THP-1 differentiation: monocytes (t = -8 h), and 2 and 16 hour post-LPS stimulated. The 2 h post-IFNγ / LPS stimulation was chosen in addition to the monocytic and 16 h post-IFNγ / LPS stimulated stages of THP-1 differentiation because the mRNA expression of RAA down-regulated mRNAs usually increased at this time point.

An example of mRNA half-life determination is shown for IL6 mRNA. The IL6 mRNA remaining in the presence of the transcriptional inhibitor, ActD, as a function of time in 2 and 16 h post-LPS stimulated THP-1 cells is shown in Figure 3.7. The mRNA decay graph for THP-1 monocytes was not shown because IL6 mRNA was not expressed. Copy number per microgram RNA, which had been converted from the real-time RT-PCR results of diluted first strand cDNA, was plotted against ActD incubation time. In the absence of ActD (control), the steady-state level of mRNA increased over the time course of the 4 hour study, indicating on-going transcription.

At 2 h post-LPS stimulation, the half-life of IL6 mRNA is 164 min in the presence of ActD, but decreases substantially to 12 min when treated with both ActD and RAA. Similarly in 16 h post-LPS stimulated cells, the half-life is 182 min in the presence of ActD, but is shortened to 17 min in the presence of both ActD and RAA. All mRNA half-lives were calculated based on the initial rate of decay (t < 60 min). Addition of ActD would determine whether RAA down-regulated mRNAs at the post-transcriptional level. However, the contribution of
the transcription rate to the RAA-induced down-regulation on mRNA steady-state levels is unknown.

The decay of IL6 mRNA shows biphasic kinetics in the presence of RAA and ActD, or even ActD alone (Fig. 3.7). There is an initial rapid decrease in the mRNA level which then levels off after 60 min. This effect may be due to limited real-time RT-PCR sensitivity at very low levels of mRNA; however, the biphasic decay was also observed at much higher mRNA concentrations in copy number at 16 h post-LPS stimulation. Since greater than 98 % of the mRNA population was degraded within the first 60 minutes as shown in Figure 3.7, the second, more stable species, was of very low abundance (approximately < 2 %). Thus, the plateau does not appear to be an artifact of the real-time RT-PCR, but rather a phenomenon of mRNA degradation. In fact, all of the mRNAs examined with half-lives less than 4 hours, showed biphasic kinetics.
Figure 3.7. Decay of IL6 mRNA in IFNγ / LPS-stimulated THP-1 cells.

Total RNA was harvested at 0, 15, 45, 75, 135, and 240 minutes in (A) 2 h or (B) 16 h post-LPS stimulated THP-1 cells treated with (△) ActD (5 µg / mL), (○) ActD (5 µg / mL) + RAA (1 µM), or (●) control vehicle (methanol + DMSO). Copy number per µg RNA was plotted against ActD incubation time. Copy number was calculated from a SNAPC2 real-time PCR amplicon standard curve based on the method of Ovstebo et al. (103).
3.6.4 mRNA Half-Life Versus the Stage of THP-1 Differentiation

Table 3.4 shows a summary of the mRNA half-lives of the genes listed in Table 3.3, in monocytes, 2 and 16 hour post-INFγ / LPS stimulated THP-1 cells. mRNA half-lives were calculated based on a linear regression fit of mRNA levels within the first 60 minutes (the initial rate of decay), and also from 75 to 240 minutes (the lag rate of decay). Only mRNA half-lives obtained from the initial decay were used for analysis because mRNA half-lives measured at the lag decay did not vary much, and were prolonged to an average over 180 minutes in all three stages of THP-1 differentiation.

As shown in Table 3.4, in the monocytic stage, only the half-life for IL-1β mRNA in the presence of RAA had a change greater than 1.5-fold (t½ = 47 min versus 25 min), indicating destabilization of mRNA by RAA at the post-transcriptional level. In both 2 and 16 h post-INFγ / LPS stimulated THP-1 cells, half-lives of the SK box-containing mRNAs: CCL4, IL6, IL1β, IL10RA, and COX2 were destabilized at the post-transcriptional level by RAA. Half-lives of NFKBIA and TNF-α mRNAs, which contain 3 and 9 SK boxes, respectively, did not change upon RAA treatment, suggesting that RAA could also modulate steady-state mRNA levels at the level of transcription. mRNAs without SK boxes, TMSB4X, KYNU, and CCL5, had half-lives longer than 4 hours at all three stages of THP-1 differentiation measured. When mRNA half-life was longer than the 4 hour course of study, its half-life was indicated as being longer than 4 hours (>4 h) rather than stating the extrapolated values. As shown in Table 3.2, the fold decrease of these SK box-lacking mRNAs was also modest among all the RAA down-regulated mRNAs.
Comparing the mRNA half-lives (with ActD treatment alone) of monocytes to 2 and 16 h post-LPS stimulated THP-1 cells, longer half-lives were observed in IL-1β, IL10RA, and COX2 mRNAs, indicating that mRNAs were stabilized upon IFNγ / LPS stimulation (see Table 3.4). Even though IL6 mRNA was not expressed in THP-1 monocytes in this study, its half-life after 1 h LPS stimulation in human peripheral blood monocytes was 30 minutes (89). Assuming that IL6 mRNA would have a matching value in mRNA half-life in THP-1 monocytes, the half-life of IL6 mRNA was stabilized to 164 and 182 min in 2 and 16 h post-LPS stimulated THP-1 cells, respectively. Therefore, all mRNAs stabilized during THP-1 cell differentiation upon IFNγ / LPS challenge (IL6, IL-1β, IL10RA, and COX2) were sensitive to RAA at the mRNA half-life level. The half-life of NFKBIA and TNF-α mRNAs (with ActD alone) remained unchanged across all three stages of THP-1 differentiation. Only CCL4 mRNA (with ActD alone) had an obviously shortened half-life when THP-1 monocytes matured to 2 and 16 h post-LPS stimulated THP-1 cells. Another interesting finding is that RAA shortened mRNA half-lives of IL6, IL-1β, TNF-α, and COX2, to an average of 10-12 minutes in 2 h post-LPS stimulated THP-1 cells.
<table>
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<tr>
<th>mRNA</th>
<th>Monocytes</th>
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<th>16 h post-LPS</th>
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<tr>
<td></td>
<td>ActD</td>
<td>ActD + RAA</td>
<td>Ratio of ActD to ActD + RAA</td>
</tr>
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<td>CCL4</td>
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<td>NE</td>
</tr>
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</tr>
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<td>39.9</td>
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</tr>
<tr>
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<td>&gt; 4h</td>
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<td>CCL5</td>
<td>&gt; 4h</td>
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</table>

Table 3.4. mRNA half-lives at 3 stages of THP-1 cell differentiation.

Half-lives were calculated based on linear regression of mRNA levels within the first 60 minutes—the initial rate of decay. Ratios of ActD to ActD+RAA highlighted in grey indicate changes ≥ 1.5-fold. *NE, not expressed. IL6 mRNA half-life in 1 h post-LPS stimulated human peripheral blood monocytes was 30 minutes (89).
3.6.5 Effect of RAA Versus the Stage of THP-1 Differentiation

Data from the mRNA half-life studies at all three stages of THP-1 differentiation also revealed the effect of RAA (4 h treatment) on mRNA stability. The effect of RAA (8 h treatment) on mRNA down-regulation was already measured (in the SAGE Validation section) in 16 h post-LPS stimulated THP-1 cells. In addition to the 16 h post-LPS stimulation, the monocytic and 2 h post-LPS stimulated THP-1 cells would give extra information to characterize the effect of RAA (4 h treatment) on mRNA destabilization by comparing the change in mRNA expression across these three stages of THP-1 differentiation.

As shown in Figure 3.8 A and Table 3.5, RAA significantly down-regulated IL-1β and TNF-α mRNAs in monocytic THP-1 cells. Although both IL-1β (6 SK boxes) and TNF-α (9 SK boxes) mRNAs belong to ARED clustering Group I (listed in Table 3.3), only the half-life of IL-1β mRNA was affected by RAA (reduced 1.9 fold). mRNAs for CCL4, NFKBIA, IL6, IL10RA, COX2, TMSB4X, KYNU, and CCL5, were unaffected by RAA at the monocytic stage. All mRNAs were down-regulated in 2 and 16 h post-LPS stimulated THP-1 cells. The effect of RAA on these mRNAs appeared to correlate to the IFNγ / LPS stimulation in THP-1 cell differentiation that they were down-regulated by RAA only after IFNγ / LPS stimulation.

The degree of RAA down-regulation (4 h treatment) at 2 and 16 h post-LPS stimulation (Figure 3.8 B and C, and Table 3.5) were usually similar but not identical because the pattern of mRNA expression began at different times, as shown in Figure 3.5 A to C. However,
CCL4, IL6, and COX2 mRNAs were the exceptions that their expression levels varied much between 2 and 16 h post-LPS stimulation. CCL4, IL6, IL-1β, and TNF-α mRNAs were down-regulated greatly (> 20 fold decrease) at these two stages of THP-1 differentiation, whereas NFKBIA and IL10RA mRNAs were down-regulated not as much relatively. COX2 mRNA was a special case that its mRNA steady-state level was strongly reduced from 8 fold decrease at 2 h post-LPS stimulation to 480 fold decrease at 16 h post-LPS stimulation. TMSB4X, KYNU, and CCL5 mRNAs exhibited very modest effects of RAA-mediated mRNA down-regulation. Differences in copy number among samples might be magnified after multiplying to a larger amount of total RNA because the copy number per microgram total RNA was converted from the real-time RT-PCR results of diluted first strand cDNA. For instance, as shown in Figure 3.8 A, IL6 mRNA had 0.05 (control) and 0.12 (RAA) copy number per 1:8 diluted 1 μL of first strand cDNA in THP-1 monocytes, but were converted to 14 and 31 copy number per microgram total RNA, respectively. Therefore, the fold decrease in lowly expressed mRNAs, such as IL6 mRNA in monocytes (Table 3.5) did not reflect the actual changes in mRNA expression.
A. THP-1 monocytes (t = -8 h)

B. 2 hour post-INFγ / LPS stimulated THP-1 cells
C. 16 hour post-INFγ / LPS stimulated THP-1 cells

Figure 3.8. The effect of RAA on the steady-state mRNA levels in (A) monocytic, (B) 2 hour, and (C) 16 hour post-IFNγ / LPS stimulated THP-1 cells.

mRNA copy number of the genes listed in Table 3.4 were determined after a 4 hour treatment of RAA (1μM) and compared to control. Copy number per RNA (microgram) was calculated from a SNAPC2 real-time PCR amplicon standard curve based on the method of Ovstebo *et al.* (103).
Table 3.5. The effect of RAA on the steady-state mRNA levels in monocytic, 2 hour and 16 hour post-IFNγ / LPS stimulated THP-1 cells.

This is a numerical representation of Figure 3.8. mRNA copy number of the genes listed in Table 3.4 were determined after a 4 hour treatment of RAA (1 µM) and compared to control. Copy number per µg RNA was calculated from a SNAPC2 real-time PCR amplicon standard curve based on the method of Ovstebo et al. (103). Fold decrease was calculated using the copy number found in untreated cells against the copy number found in RAA-treated cells.
4.0 Discussion

4.1 SAGE Data: Specificity of the Action of RAA on mRNA Down-Regulation

This research project presents two ways of examining the effect of RAA on mRNA levels in THP-1 cells – SAGE and real-time RT-PCR. Data from two ~45,000-tag SAGE libraries of THP-1 cells stimulated with IFNγ / LPS and treated in the absence or presence of RAA (1 μM) were used to investigate the effect of RAA at a transcriptome-wide level. Real-time RT-PCR was used as a complementary method to validate SAGE data. Despite its power to survey the entire transcriptome, SAGE loses its power to detect low-expression mRNAs when less than 50,000 tags were sequenced. mRNAs such as IL-1α, IL6, and COX2 were not found in SAGE data, but were found to be strongly sensitive to RAA in Kastelic et al. (64) and when examined using real-time RT-PCR in this study. Nonetheless, the effect of RAA on mRNAs in THP-1 cells is potent and specific because only 0.27 % of the 17,608 unique tags detected were down-regulated based on the SAGE data (p value < 0.001).

The total unassigned tags from the 800 SAGE tags analyzed (first 400 down-regulated, and first 400 up-regulated SAGE tags, discussed in section 3.1) were 10.8 %, excluding ambiguous tag-to-gene identity assignments. Most of the unmatched tags were found in low abundance (tag counts < 6). The number of unmatched tags appeared to be less than the literature values reported by Chen et al. (31) in the range of 50 to 70 % because only the first 800 of the most abundant tags were examined instead of the full reservoir of unique tags, ambiguous tag-to-gene identity assignments were excluded, and different species were examined. Of these 800 tags queried with the ARED online database (9), more down-regulated tags (8.5 %) appeared to encode SK box-containing mRNAs, comparing to the up-
regulated ones (1.5 %). Compared to the average expression level of ARE-containing mRNAs at about 5-8 % of human genes (10), fewer mRNAs containing SK boxes were found in the RAA up-regulated tags based on the SAGE data. The mRNA up-regulation could possibly be the result of down-regulation of certain regulatory mRNAs upon RAA treatment such that these up-regulated mRNAs might be a secondary consequence. Astonishingly, 73 % of the mRNAs down-regulated (> 1.5-fold, p value < 0.001) by RAA contained SK boxes.

4.2 Antisense mRNA Expression

Of the 48 down-regulated tags (at a confidence level of 99.9 %), 9 unmatched tags (19 %) appeared to encode antisense transcripts. In recent literature, at least 8 % of the total human gene expression was estimated to be antisense transcripts (24), and some could have expression even as high as 22 % (30) of the total gene expression. In SAGE libraries, more antisense tags were usually found at low abundance, especially among singletons (44). As shown in the Results section 3.4, the expression level of antisense transcripts in real-time RT-PCR results had a great discrepancy from the SAGE data. SAGE tags encoding antisense mRNA in the untreated library appeared to have higher expression than the expression level measured using real-time RT-PCR. Moreover, the level of antisense mRNAs was not affected by the RAA treatment based on the real-time RT-PCR results, in contrast to the strong down-regulation revealed by the SAGE data. Although results from real-time RT-PCR confirmed the expression of antisense transcripts, the mRNA level resulting from the effect of RAA was inconsistent. The disagreement may be due to the inaccurate measurement of the antisense mRNA level using the current method and primer pairs.
The presence of antisense transcripts may have varied effects on exploring the transcriptome. Hybridization of sense and their corresponding antisense transcripts may prevent isolation of mRNA for cDNA synthesis. Consequently, sense and antisense transcripts may be missing from both cDNA and SAGE libraries. Also, a large number of the low abundance tags mapped to antisense transcripts of more abundant sense transcripts. Therefore, the gene number reported in SAGE analysis could possibly be an overestimate, due to the fact that the cells use the complexity of transcriptome (including multiple effects of antisense transcription, alternative polyadenylation, splicing and cleavage site selection, etc.) to fine-tune their functions.

4.3 Real-time RT-PCR Versus SAGE Technology

The real-time quantitative PCR analyses confirmed the tag-to-gene identities determined for the two ~45,000-tag SAGE libraries of THP-1 cells (t = 24 h) stimulated with IFNγ / LPS and treated ± RAA (1 μM), as well as quantified the mRNA expression. Fold decreases in SAGE tags, from libraries that had not been sequenced to over 100,000 tags, might not reflect the actual degree of RAA down-regulation. Results from real-time RT-PCR revealed that only 8 mRNAs, CCL2, CCL3, CCL4, CCL8, IL-1α, IL-1β, IL6, and IL8, had much less fold decreases compared to the SAGE data (see Table 3.2) upon RAA treatment. Taking CCL4 mRNA as an example, it was found to be 12.5-fold decreased based on real-time RT-PCR, but 492-fold decreased in SAGE data. However, the reliability of the tag count in SAGE is inconsistent as reported in the literature. In the studies of Hashimoto et al. (51), Short- and Long-SAGE libraries (all normalized to 35,800 total tags) were constructed from
Peripheral blood monocytes stimulated with LPS for 3 hours. Both libraries, prepared in the same manner, had 3 tags mapped to CCL4 mRNA (51): 367 LongSAGE tags against 215 ShortSAGE tags (ratio = 1.7); 361 against 318 (ratio = 1.1); and 100 against 11 (ratio = 9.1). Results from the studies of Hashimoto et al. (51) showed that the frequency of SAGE tags encoding the same mRNA varied considerably, especially in the ShortSAGE library with less than 50,000 tags sequenced (168). Therefore, validation of SAGE data using a complementary approach, such as real-time RT-PCR, is necessary to confirm the mRNA identity and its expression level. Nevertheless, in this study, fold decreases in mRNA expression level from the real-time RT-PCR results generally showed good correlation with the SAGE data (see Table 3.2).

4.4 Optimal Actinomycin D Concentration & mRNA Half-Life Studies for THP-1 Cells

An optimal ActD concentration for THP-1 cells was needed to inhibit mRNA transcription maximally while maintaining survival of most cells. The optimal ActD concentration determined for THP-1 cells was 5 μg / mL, which is comparable to that described in the literature for various cell lines. Early studies by Perry (108), and Sawicki and Godman (128) showed that 0.04 μg / mL ActD could inhibit more than half of the transcription. However, at this ActD concentration, and even up to 1.0 μg / mL in this work, transcriptional inhibition was not sustained over the 4 hour period of half-life study. The established LD50 for actinomycin D in undifferentiated THP-1 was 120 ng / mL for 24 h, measured using FACS with propidium iodine stain (110). Since the purpose of the mRNA half-life study was to determine whether RAA down-regulated mRNA at the post-transcriptional level, 5.0 μg / mL
ActD was selected to inhibit mRNA transcription completely (98 % inhibition) for THP-1 cells over the 4 hour of study.

Quantification of these mRNA half-lives revealed a biphasic pattern with initial rapid first-order kinetics, followed by a plateau at less than 2 % of the initial mRNA levels (Figure 3.7). After the first 60 minutes of ActD addition, all mRNAs decayed slowly with an average lag half-life of 180 minutes. This arrest of degradation (in a plateauing-off fashion) is not an artifact but most likely a general effect attributed to the transcription inhibitor and also mRNA degradation (142, 150). First, the maximal transcriptional inhibition in this study using 5.0 μg / mL ActD was about 98 %; therefore, the observed plateau could possibly reflect the uninhibited portion (< 2 %). Secondly, the rapid ARE-mediated mRNA degrading pathway may explain the biphasic kinetics. Once all less stable ARE-containing mRNAs have decayed, only isoforms of those ARE-lacking mRNAs are left behind.

4.5 RAA and its Target of Action

Unexpectedly, the fold decrease in mRNA expression levels upon RAA treatment did not correlate with the number of SK boxes, based on the real-time RT-PCR results. However, as indicated by the two ARE classifications, the organization of SK boxes rather than their number, was a more substantial determinant in mRNA degradation. Moreover, as shown in Kastelic et al. (64), RAA-mediated mRNA down-regulation was sufficiently triggered by a 30 bp stretch which contained the ARED clustering Group 1, in the 3’ UTR of IL-1β in a luciferase gene expression assay. Therefore, specific features on the organization of SK
boxes of the ARED clustering Group 1 might possibly be responsible to the mRNA sensitivity to RAA.

In order to further correlate the SK box organization with the effect of RAA on mRNA half-life, 10 representative RAA-sensitive mRNAs were chosen based on their ARE class. Three mRNAs without SK boxes, TMSB4X, KYNU, and CCL5, had half-lives greater than 4 hours in all three stages of THP-1 differentiation. The observation in mRNA half-life agreed with the literature that SK box-lacking mRNAs had longer half-lives, usually in the 10-12 hour range (76, 119). All 3 mRNAs were found to be mildly down-regulated by RAA from both the SAGE data and real-time RT-PCR results. However, in the study of Kastelic et al. (64), Northern blot analysis of CCL5 / RANTES mRNA showed no sensitivity toward RAA treatment. Therefore, the sensitivity of real-time RT-PCR may detect smaller changes in mRNA destabilization upon RAA treatment than Northern Blot analysis.

In both 2 and 16 h post-INFγ / LPS stimulated THP-1 cells, half-lives of 5 SK box-containing mRNAs (out of 7 SK box-containing mRNAs tested in half-life study), encoding CCL4, IL6, IL-1β, IL10RA, and COX2 mRNAs, were dramatically destabilized at the post-transcriptional level by RAA. 4 out of these 5 mRNAs (except CCL4 mRNA) were stabilized after IFNγ / LPS stimulation. Even though CCL4 mRNA was down-regulated at the mRNA steady-state level, and was affected at the post-transcriptional level upon RAA treatment, its mRNA was not stabilized when THP-1 cells were stimulated with IFNγ / LPS (with ActD alone, Table 3.4). During THP-1 cell differentiation (IFNγ / LPS), RAA may target either a pathway that stabilizes a few sets of genes, or more than one pathway that each
stabilizes a set of genes. RAA could possibly modulate mRNA steady-state levels of NFKBIA and TNF-α at the transcriptional level because their mRNAs were down-regulated by RAA, yet no change was observed in their half-lives. Furthermore, based on real-time RT-PCR results, fewer mRNAs appeared to be down-regulated by RAA in THP-1 monocytes (Fig. 3.8 A), suggesting that little effect of RAA might be observed due to lower mRNA expression in monocytes, or fewer pathways might have been activated in monocytes that would be affected by RAA.

From the mRNA half-life study, the shortest average mRNA half-life was about 10-12 minutes, found in IL6, IL-1β, and TNF-α mRNAs at 2 h post-LPS stimulation upon RAA treatment. This value may reflect the default rate of mRNA decay in cells. Similar mRNA half-life values (about 10-12 minutes) have been reported in the literature (81). It could possibly result from either removing the protection / stabilization mechanisms, or triggering a destabilizing mechanism on mRNA stability by RAA.

4.6 Potential Pathways RAA Could Affect

The mRNA half-life study also pointed to a number of interesting new findings about RAA. In a recent report, Frevel et al. (39), through a bioinformatics approach, identified a set of mRNAs which were stabilized by the p38 MAPK pathway upon 2 h LPS stimulation (no IFNγ) in THP-1 cells. Both cDNA microarray and Northern blot analysis were used to measure mRNA half-lives from cells treated with 5.0 μg / mL ActD under similar conditions to this study. For example, the half-life of CCL4 mRNA reported in Frevel et al. (39) was 119 min agreeing closely with 132 min in our study. Moreover, when comparing the degree
of mRNA down-regulation, RAA appeared to exert a stronger and broader mRNA destabilizing effect than did SB203580, a p38 MAPK pathway inhibitor. For example, mRNAs, which were not down-regulated in 2 h post-LPS stimulated THP-1 cells treated with SB203580 in Frevel et al (39), were strongly down-regulated in 2 h post-IFNγ / LPS stimulated THP-1 cells treated with RAA in our study even though the SB203580-down-regulated mRNAs were affected by RAA as well. However, our study could not address the suggestion from the studies of Takehana et al. (157) that RAA’s activity did not involve the p38 MAPK pathway because we used different conditions and concentrations of RAA.

As shown by Kotlyarov et al. (70), neither TNF-α mRNA level nor TNF-α secretion were regulated by p38 MAPK. However, studies of the promoter region of TNF-α indicated that interactions of transcription factors, such as AP-1, and NF-κB, could contribute to the transcriptional regulation of TNF-α (81, 163). Takehana et al. (157) also reported that RAA only inhibited AP-1-dependent, but not NF-κB- and glucocorticoid-dependent transcriptional activity. All these results suggest that RAA may down-regulate the transcription of TNF-α by inhibiting AP-1 or other transcription factors complexes.

4.7 Comparison between RAA, Radicicol, and LL-Z

RAA is structurally much closer to LL-Z than it is to radicicol. LL-Z was found to specifically inhibit TAK1, which is a key player in regulating the NF-κB, JNK, and p38 MAPK pathways. RAA may possibly act on an upstream target of pathways, like the target of LL-Z. Furthermore, slight changes in the chemical structure of LL-Z could yield various levels of inhibitory activity on the same target, as indicated in studies of Ninomiya-Tsuji et
Regardless of the resemblance in structure, RAA showed very distinguishable effects from LL-Z in past studies (157).

Pathway specificity could be dose-dependent too. As shown in studies of Matsuoka et al. (93), ERK MAPK pathway could also be suppressed with higher concentration of LL-Z. The possibility that RAA may target something completely dissimilar from the target of LL-Z should not be neglected. No other radicicol analogue was found to block IL-1β secretion with a concomitant effect on cytokine mRNA stability. No literature on mRNA stability modulated by LL-Z has been published to date. As demonstrated by Takehana et al. (157), LL-Z was stimulation- and cell type-specific as shown by the fact that it did not inhibit TNF-induced activation of the JNK pathway in HeLa cells. Similarly, RAA may inhibit pathways triggered only by certain stimulations such as IFNγ / LPS.

4.8 Summary

The majority of tags from two SAGE libraries of THP-1 cells (t = 24 h) stimulated with IFNγ / LPS ± RAA (1 μM) was of low abundance. Low-expression mRNAs, which were significantly down-regulated by RAA, could not be detected by ~45,000-tag SAGE libraries. Thus, using real-time RT-PCR as a complementary approach, SAGE data was validated, and mRNA expression, especially low copy number expression, was quantified.

The effect of RAA is specific: based on the SAGE data, RAA-treated THP-1 cells (t = 24 h, stimulated with IFNγ / LPS) had only 0.27 % mRNAs down-regulated and 0.16 % up-regulated (p value < 0.001). Of these down-regulated mRNAs, 73 % contained SK boxes (>
1.5-fold). Unexpectedly, RAA could also down-regulate mRNAs lacking the SK box. Furthermore, based on the SAGE data, more RAA-down-regulated mRNAs contain SK boxes (8.5 %) than the up-regulated ones (1.5 %). Even though the presence of SK boxes did not correlate with RAA sensitivity, mRNA stabilization upon IFNγ / LPS stimulation correlated better. All mRNAs stabilized during THP-1 cell differentiation (IFNγ / LPS) were sensitive to RAA at the post-transcriptional level. For the first time, RAA was found to down-regulate mRNAs in THP-1 monocytes. In unstimulated monocytic THP-1 cells, only TNF-α and IL-1β mRNAs, both belonging to the ARED clustering Group I, were down-regulated by RAA. The effect of RAA on IL-1β mRNA was at the level of mRNA stability. However, TNF-α mRNA steady-state level could possibly be modulated by RAA at the transcription level because TNF-α mRNA was down-regulated by RAA, yet no change was observed in the half-life. This finding was very surprising since we anticipated that TNF-α mRNA would be down-regulated at the post-transcriptional level, similar to IL-1β mRNA.

This work shows that it is possible to affect mRNA stabilization using RAA, a small molecular weight compound. Through characterizing the effect of RAA on mRNA down-regulation in a leukemic cell line, we hope to shed light on providing new avenues of cancer research.
5.0 Future Work

Using real-time RT-PCR, this research project has validated the SAGE data (p value < 0.001), which were used to investigate RAA's effect at a transcriptome-wide level, from two ~45,000-tag SAGE libraries of THP-1 cells stimulated with IFNγ / LPS and treated in the absence or presence of RAA (1 μM). Bioinformatics analysis on SAGE data, and quantitation of RAA-down-regulated mRNAs (p value < 0.001) and their half-lives were also performed.

Determining the effect of RAA on transcription would be the first priority because the mRNA half-life study only revealed a post-transcriptional effect of RAA. A nuclear run-on transcription assay could be used to measure transcription rates in a cell nucleus at a specific time. mRNA transcripts incorporated with radiolabeled nucleotides are produced from the stalled RNA polymerase reactions at the time the cells are lysed. Then, purified mRNA is hybridized to cDNA on a membrane and analyzed via autoradiography. By comparing the amount of gene-specific radiolabeled RNA synthesized in the untreated THP-1 cells with the one treated with RAA, the effect of RAA on transcriptional initiation events could be determined.

As our research study suggests that RAA may affect more than one target in a pathway, potential targets of RAA from each pathway could be determined using cDNA microarrays, such as the GEArray. Labeled cDNAs are specifically hybridized to the surface of pre-made arrays (spotted with specific sets of genes). Then, experimental data collected by a specialized fluorescent scanner would be quantified and analyzed with further mathematical
analysis (such as clustering), and connected to the available knowledge, which may provide insights into functional pathways or common regulatory mechanisms. Gene arrays for autoimmune and inflammatory response, apoptosis and cell cycle, cancer drug resistance and metabolism, EGF / PDGF signaling pathway, extracellular matrix and adhesion molecules, G-protein coupled receptors signaling, inflammatory cytokines and receptors, JAK / STAT signaling pathway, PI3K-AKT signaling, Toll-like receptor signaling pathway, and Wnt signaling pathway, would be recommended pathways for further studies.

Further, in unstimulated monocytic THP-1 cells, only TNF-α and IL-1β mRNAs, both belonging to the ARED clustering Group I, were down-regulated by RAA. It is unclear whether possession of the ARED clustering Group I correlates with RAA-induced mRNA down-regulation in THP-1 monocytes, even though mRNA destabilization was found to be conferred by the 30 bp stretch in the 3' UTR of IL-1β which contained the ARED clustering Group 1, in THP-1 cells stimulated with IFNγ / LPS (64). This interesting aspect of the effect of RAA in THP-1 monocytes could be examined using point mutation and SK box cassette deletion within the 30 bp stretch. The minimum motif on SK box-containing mRNAs required for sufficient RAA down-regulation could be delimited by these approaches.

Next, the effect of RAA on a number of different human cancer cell lines could be studied to investigate RAA’s effect on other signal transduction pathways. Examining the effect of RAA on other leukemic cell lines such as CEM, HEL-92, and HL60, is important in order to confirm that THP-1 leukemic monocyte cell line is not the only leukemic cell line that
responds to RAA. It could be of interest to determine if the same targets/pathways are affected or if cell types have devised other techniques to influence mRNA stability. After the potency of RAA has been confirmed in other cancer cell lines, mRNA stability and its role in the initiation and progression of cancer could be studied in an animal model system.

At a later stage of the research, if the target of RAA has been confirmed, a specifically designed siRNA which knocks out the target of RAA could be used to determine whether it is the exclusive target of RAA. Then, the potential active site of the target inhibited by RAA could be elucidated by nuclear magnetic resonance (NMR) and X-ray crystallography. NMR analysis would determine whether RAA ligand binding has distinct direct interactions and conformational rearrangements. Structures of the target in complex with RAA, radicicol, and LL-Z could be examined using X-ray crystallography.

Eventually, data from this project will result in a more detailed understanding of the mechanisms by which mRNAs are regulated at the transcriptional and post-transcriptional level. Possible RAA-affected pathways and specific mRNA instability determinants in THP-1 monocytes could be elucidated. In this manner, we hope to translate the research on RAA to clinical application in humans.
6.0 Bibliography


### 7.0 Appendices

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This table summarizes the 37 mRNAs plotted on Figure 3.3 A and B. mRNAs are ranked according to fold decrease in real-time RT-PCR results.