UNIVERSITY OF CALIFORNIA, SAN DIEGO

Expression of Eukaryotic Translation Initiation Factors in murine B cells treated with 33 single ligands

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

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Professor Xiaohua Huang

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The thesis of Raj Srikrishnan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Expression of Eukaryotic Translation Initiation Factors in murine B cells treated with 33 single ligands

by

Raj Srikrishnan

Master of Science in Bioengineering

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Professor Shankar Subramaniam, Chair

Eukaryotic Translation Initiation is governed by large initiation factor complexes, whose subunits are regulated in a largely discordant fashion. The Alliance for Cellular Signaling (AfCS) murine B cell single ligand screen with 33 ligands utilizing Agilent microarrays was analyzed for its effects on initiation factor gene expression. These ligands included those that stimulate receptor classes such as GPCR, kinase receptors, TNF superfamily receptors and immunoglobulin receptors. These receptors act in pathways such as NFκB, STAT6, JNK, ERK, PI(3)K, GPCR, cytokine signaling and interferon signaling. eIF2, which is

inactivated upon phosphorylation of subunit 1 by α kinases 1-4, was seen to have discordant regulation of subunits and differing levels of kinase gene expression for different ligands, indicating a high degree of variability in global translation. eIF3, a large complex with at least 10 subunits, is seen to have discordant regulation of its subunits as well, even for a conserved ‘core’ complex, attesting to their dual functions in cellular machinery. eIF4, a complex consisting of 3 subunits with multiple subunit isoforms, was seen to express only eIF4G isoform 3 in murine B cells. eIF4A expression was limited to two isoforms, eIF4A and eIF4A3. Similar to eIF2s1, eIF4E can be phosphorylated by binding proteins, and these are seen to have differing levels of gene expression with various ligand stimulations, allowing for large variability in protein synthesis.

Introduction

B cells:

In this study, expression profiles of eukaryotic translation initiation factors were investigated using murine B cells treated individually with 33 ligands that are known to influence several cellular processes. These processes include those involved in inflammatory response, cell adhesion, cell migration, growth and apoptosis. B cells are a key component of the humoral immune system and constitute a class of cells known as lymphocytes. More commonly these cells are referred to as white blood cells (WBCs). They are derived from hematopoietic stem cell progenitors, which also give rise to other lymphocytes and erythrocytes, as shown in Figure 1. Reticulocytes differentiate into erythrocytes, better known as red blood cells that carry hemoglobin and are primarily responsible for the transport of oxygen in the blood.

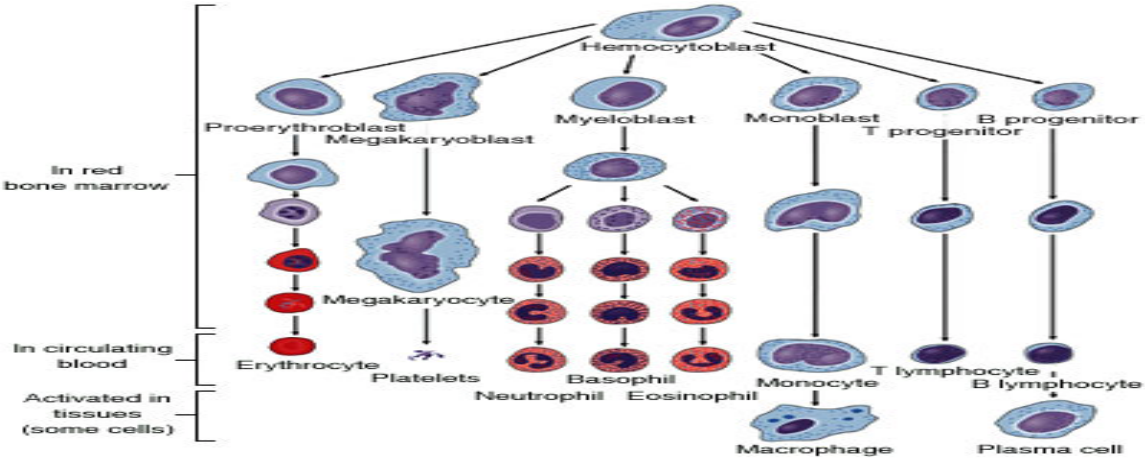


Figure 1 - Hematopoietic Stem Cell lineage

The remainder of the cells in the hematopoietic system are involved in immunologic responses, and play significant roles in phagocytosis and antigen presentation. In the immune system B cells are involved in antibody production as well as function as antigen presenting cells (APCs). They end-differentiate into memory B cells (also known as plasma cells) following activation by an interacting antigen. Immature B cells are produced in the bone marrow and then migrate to the spleen where they differentiate into mature B lymphocytes. B cells differentiate by a process illustrated in Figure 2, that is characterized by the development of antibodies. Antibodies comprise 2 heavy chains and 2 light chains. The light chains are specified by genes found in the variable (V) region,

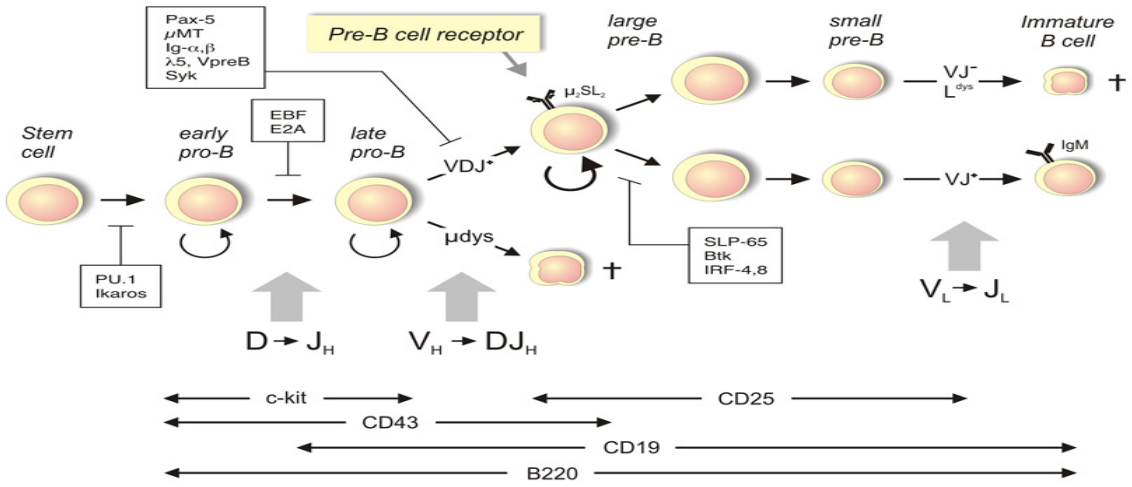


Figure 2 - B cell development from stem cell progenitor to immature B cell (classified as a mature B cell upon encountering antigen) adopted from

http://www.biologie.uni-erlangen.de/genetik/resact_winkler.php

as well as the constant (C) region. Heavy chains are composed of V and C segments, as well as a diversity (D) segment. These recombine randomly in a process known as VDJ recombination, which is responsible for the vast diversity of epitopes recognized by B cell receptors. B cells commit to cell suicide through

a mechanism known as apoptosis when steps in the maturation process fail. (Parham, 2005) Apoptosis induction also occurs if the antigen recognized by the B cell receptor (BCR) is present in the host organism. However, when a foreign antigen is detected to match the epitope recognized by the BCR, the B cell is induced to proliferate and secrete the BCR as antibodies. (Allman *et al*, 2004)

Ligands:

The process by which ligands to the known receptors on B cells act to influence the cellular machinery is complex. These ligands can be broadly categorized to induce B cell pathways involved in activation, proliferation, migration, differentiation, isotype class switching, somatic hypermutation, anergy and apoptosis, among others. Ligands known to induce or co-stimulate proliferation include anti-Ig, CD40L, LPS, IL-4 and CpG. Another group, broadly classified as chemotactic ligands, includes BLC, ELC, SLC, fMLP, MIP3a and SDF1, which mediate B cell migration through the B lymphocyte maturation process. (Zhu *et al*, 2004) The pathways affected include the NFκB pathway, STAT6, JNK, ERK, PI(3)K, GPCR, cytokine signaling and interferon signaling (Natarajan *et al*, 2006). These ligands stimulate receptors classes such as GPCR, kinase receptors, the TNF superfamily receptors and immunoglobulin receptors.

In a previous study, patterns of differential gene expression have shown that only CD40L, LPS, Anti-Ig, IL-4, and CpG, broadly classified as inflammatory ligands, affected 200-1800 B cell genes that were identified by Significance Analysis of

Microarrays (SAM) analysis (Lee *et al*, 2006). In this study it was also shown that the remaining 28 ligands influenced change in less than 100 genes from a chip that contained over 10, 000 unique genes.

Table 1 - The 33 Single Ligand Stimulations used in this study

Abbr	Name	B cell Function	Functional Classification	Receptors
ELC	ELC (CCL19; MIP3b)	Integrin activation/migration	Adhesion	GPCR
SLC	SLC (CCL21; 6Ckine)	Integrin activation	Adhesion	GPCR
TGF	Transforming growth factor-beta	Multiple Functions	Apoptosis	Kinase R.
70L	CD27	Activation/Ig Synthesis	Apoptosis	TNF R.
GRH	Growth hormone release hormone	Inhibit chemotactic responses	Growth	GPCR
IGF	IGF-1	Unclear	Growth	Kinase R.
S1P	Sphingosine-1-phosphate	B cell sequestration	Immunosuppr.	GPCR
TER	Terbutaline	Modulate Ig production, increase cAMP	Immunosuppr.	GPCR
I10	Interleukin-10	Proliferation, differentiation	Immunosuppr.	Kinase R.
AIG	Antigen (Anti-Ig)	Prolif., Diff., Isotype Switching	Inflammation	Antibody
LB4	LTB4 (leukotriene B4)	Unclear	Inflammation	GPCR
LPA	Lysophosphatidic acid	Prolif., Diff., Isotype Switching	Inflammation	GPCR
PAF	Platelet Activating Factor	Unclear	Inflammation	GPCR
PGE	Prostaglandin E2	Unclear	Inflammation	GPCR
DIM	Dimaprit	Inhibit IgE production	Inflammation	GPCR*
IFB	Interferon-beta	Inhibits apoptosis	Inflammation	Kinase R.
IFG	Interferon-gamma	Specific Isotype switching	Inflammation	Kinase R.
CPG	CpG-oligodeoxynucleotide	Proliferation, IL6/IL10/Antibody Secretion	Inflammation	TLR
LPS	Lipopolysaccharide	Proliferation	Inflammation	TLR
40L	CD40L (CD154)	Prolif., Diff., Isotype Switching	Inflammation	TNF R.
BAF	BAFF (BlyS)	Prolif., Diff., Isotype Switching	Inflammation	TNF R.
TNF	TNF alpha	Multiple Functions	Inflammation	TNF R.
I04	Interleukin-4	Prolif., Diff., Isotype Switching	Inflammation	
BLC	BLC (BCA-1, CXCL13)	Migration and Differentiation	Migration	GPCR
FML	fMLP	Chemotaxis	Migration	GPCR
M3A	MIP3a (CCl20)	Migration	Migration	GPCR
SDF	SDF1 (CXCL12)	Migration	Migration	GPCR
2MA	2-Methyl-thio-ATP	Increases Intercellular Ca++		GPCR
BOM	Bombesin	Unclear		GPCR
CGS	CGS-21680 hydrochloride(Adenosine)	Increases cAMP, apoptosis rescue		GPCR
NEB	Neurokinin B	Unclear		GPCR
NPY	Neuropeptide Y	Unclear		GPCR
NGF	NGF	Unclear		Kinase R.
SIMDM	Suppl. Iscove's Mod. Dulbecco's	Control Medium		

Eukaryotic Translation:

Table 2 - Eukaryotic Translation Initiation Factors and Entrez GeneIDs

eIF1	eIF2	eIF3	eIF4	eIF5
eIF1A(13664)	eIF Subunit 1 (13665)	eIF3 subunit a (13669)	eIF4A isoform 1 (13681)	eIF5a
eIF1A y-linked (66235)	eIF Subunit 2 (67204)	eIF3 subunit b (27979)	eIF4A isoform 2	eIF5b
eIF1B (68969)	eIF Subunit 3 (26908)	eIF3 subunit c (56347)	eIF4A isoform 3 (192170)	
		eIF3 subunit d (55944)	eIF4G isoform 3 (230861)	
	eIF2 α-kinase 1 (15467)	eIF3 subunit e (16341)	eIF4E (13684)	
	eIF2 α-kinase 2 (19106)	eIF3 subunit f (66085)	eIF4E isoform 2 (26987)	
	eIF2 α-kinase 3 (13666)	eIF3 subunit h (68135)	eIF4E isoform 3 (66892)	
	eIF2 α-kinase 4 (27103)	eIF3 subunit l (54709)		
		eIF3 subunit j (78655)	eIF4E Binding Protein 1 (13685)	
		eiF3 subunit l (223691)	eIF4E Binding Protein 2 (13688)	

Eukaryotic translation is the process whereby messenger RNA (mRNA) is translated into proteins in eukaryotes. It is characterized by three principal processes: Initiation, Elongation, and Termination. (Kapp *et al*, 2004)

Two modes of translation initiation exist: cap-dependent and the much rarer cap-independent initiation. Cap-dependent initiation involves interaction of special proteins to the 5’ cap terminus of processed mRNA. The 5’ cap is a chemically altered nucleotide at the 5’ end of mRNA, characterized by an unusual 5’ to 5’ triphosphate linkage. In addition, this last nucleotide is always a guanine base, methylated on the 7^{th} position. It is thus typically annotated as the

5’ m^7G cap, and serves as a marker of fully processed mature mRNA, along with the poly-A rich tail.

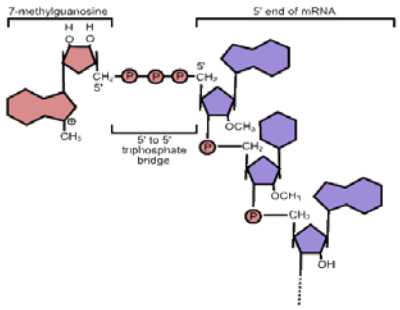


Figure 3 - 5' Cap of mRNA

(adopted from http://commons.wikimedia.org/wiki/File:5%27_cap_structure.png)

Cap-independent translation is distinguished from cap-dependent translation by the fact that the 40S ribosome does not bind the 5’ terminus and can initiate translation at an internal site. (López-Lastra *et al*, 2005) This phenomenon is termed internal initiation and the site is referred to as Internal Ribosomal Entry Site (IRES). Several RNA viral genes show IRES-mediated translation and about 300 eukaryotic genes do demonstrate putative IRES elements. (Romero-Lopez *et* al, 2005) The ribosome can be directed to the start site by IRES trans-acting factors (ITAFs). IRESes thus far identified do not show any similarity in their primary or secondary sequence.

After the translation initiation process is completed, elongation proceeds, using eukaryotic elongation factors, in a three step cycle. The first step is positioning of the correct (codon to anti-codon) charged tRNA (tRNA molecule

and bound peptide) in the A site of the 60S ribosomal subunit. The second step is characterized by the formation of the peptide bond between the P site and the A site peptides. The third step is translocation of the ribosome three nucleotides towards the 3’ end of the mRNA (the length of one codon) using GTP hydrolysis for energy.

Lastly, when a termination codon (UAA, UAG, or UGA) enters the A site, a release factor (RF1 or RF2) moves into the A site, triggering hydrolysis of the ester bond in the peptidyl-tRNA (at the P site), leading to the release of the newly synthesized protein from the ribosome. Lastly, RF3 releases RF1 and RF2 from the ribosome, freeing the ribosome recycling factor to dissociate the ribosome for subsequent rounds of translation. (López-Lastra *et al*, 2005)

Eukaryotic Translation Initiation:

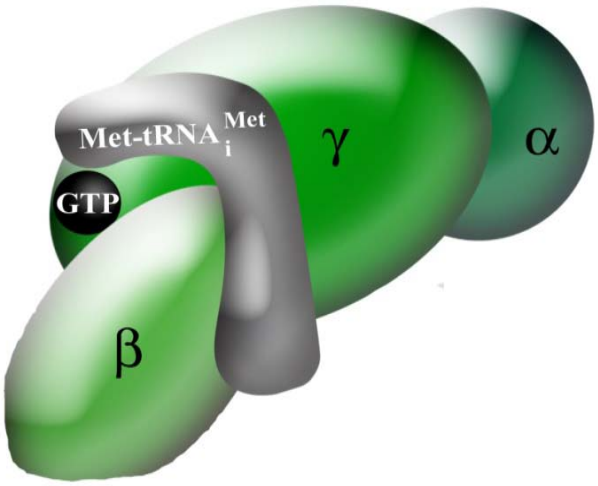


Figure 4 - Ternary Complex consisting of 3 eIF2 subunits, charged tRNA and GTP (adapted from http://www.biomed.cas.cz/mbu/lrge/research.html)

As indicated earlier eukaryotic translation initiation is a process whereby translation of the genetic information encoded in mRNA is translated into protein. It starts by the formation of the ternary complex, illustrated in Figure 4, which is composed of eIF2, charged tRNA (Methionyl-tRNA), and bound GTP. (Kimball, 1999) The factor eIF2B is involved in recycling eIF2 with GTP. It is important to note that eIF2 cannot dissociate from eIF2B, if eIF2 subunit 1 is phosphorylated by any of the eIF2 α-kinases (Alpha Kinases 1-4). This phosphorylation shuts down translation, as eIF2 cannot then form the ternary complex. (Hinnebusch, 1993)

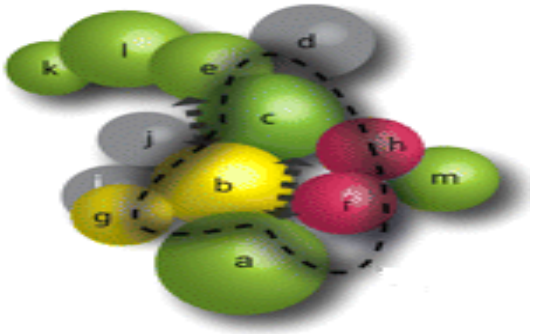


Figure 5 - Ten Subunits of eIF3 (adapted from Zhou *et al*, 2008)

This ternary complex next binds to eIF3 and the small ribosomal subunit, known as 40S. This new complex is known as the 43S complex. eIF3, illustrated in Figure 5, is a large complex of 650 kDa, consisting of between ten and fifteen subunits and these numbers vary between species. (Asano *et al*, 2007) It has been shown that translation can still proceed if a few of these are knocked out (Masutani *et al*, 2007). It has also been shown that these subunits perform dual functions (Luke-Glaser *et al*, 2007)

The eIF4F complex comprises 4 subunits (eIF4E, eIF4G, and 2 copies of eIFA subunits), as illustrated in Figure 6. (Gingras *et al*, 1999) All three subunits in eIF4F have been shown to have multiple isoforms that are expressed in a tissue specific manner. eIF4E is the 5’ cap binding factor whereas eIF4G is a scaffolding protein as it facilitates the interaction between as the cap site and 3’ untranslated region through its interaction with the PolyA-Binding protein (PABP).

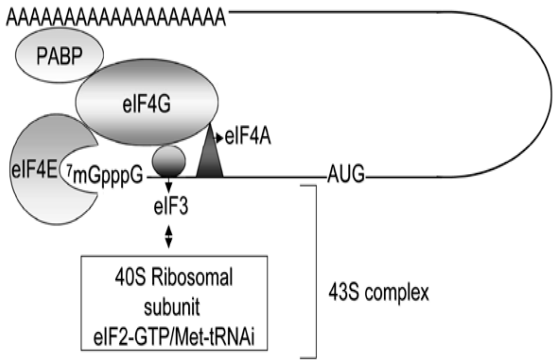


Figure 6 - eIF4F complex as part of the 48S complex

adopted from http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0716-97602005000200003&tlng=en&lng=en&nrm=iso

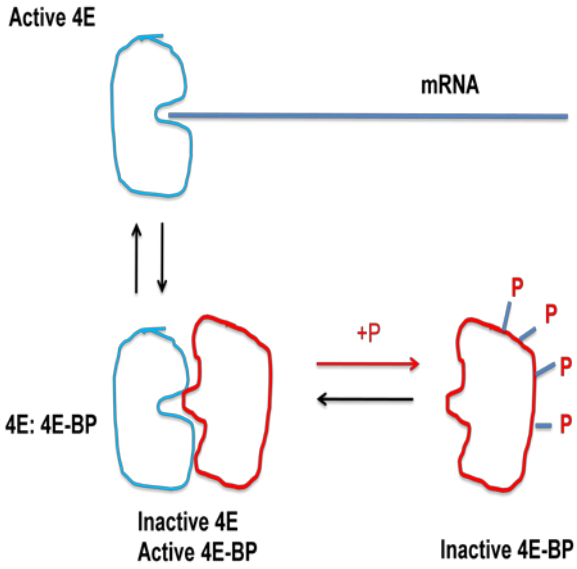


Figure 7 - eIF4E to eIF4E-BP1/2 Interaction

n addition to eIF2 α-kinases, another mechanism whereby translation can be blocked is mediated by eIF4E. The two isoforms that interact with eIF4E have been identified, namely eIF4E-Binding Protein 1 and eIF4E-Binding Protein 2. The binding of eIF4E to eIF4E-BPs prevents eIF4E from binding the cap site and thereby blocks translation. (Joshi *et al*, 2004) This interaction between eIF4E and eIF4E-BP can be blocked if the binding proteins are phosphorylated such as in

the presence of rapamycin that inhibits mTOR (the mammalian target of rapamycin) protein (Sonenberg *et al*, 2009). eIF4E is free to bind the mRNA at the 5' cap site, thus triggering translation. This process is illustrated in Figure 5.

The eIF4F complex and the mRNA molecule next bind the 43S complex, constituting the ternary complex, eIF3 and 40S ribosome, to form the 48S complex. The process of mRNA unwinding is triggered by ATP hydrolysis mediated by eIF4A. The 48S complex then scans the mRNA in search of a start codon (AUG). This scanning process is influenced by eIF1A. (Battiste *et al*, 2000) Lastly, upon finding the start codon, eIF5 causes the dissociation of all initiation factors, allowing the small (40S) ribosomal subunit to bind the larger (60S) ribosomal subunit to form the 80S complex. Translational elongation now begins in a 5' to 3' direction to synthesize the nascent polypeptide.

Materials & Methods

Experimental Methods

Resting B lymphocytes were isolated from mouse spleens and then incubated at 37 °C in media for 1 hr, followed by incubation with media and

ligands together (treatment) or just media (no treatment) at concentrations ranging from 1 nM to 0.1 µM (40 µg/ml in the case of LPS) and without ligand (no treatment) for desired times (illustrated below), and then lysed to reveal cellular contents. There were a total of 33 ligands, as listed above. mRNA was isolated using a mixture of TriPure, chloroform and isopropanol, and using a dT-primer, reverse transcribed into first strand cDNA. This was used as a template to generate second strand cDNA. This was used to generate cRNA *in vitro*. The cRNA template was used to generate a Cy-dUTP (treatment) or Cy3-dUTP (no treatment) labeled RNA for hybridization to microarray slides. Labeled cRNA was hybridized to custom Agilent mouse chips containing 16273 probes. There were a total of 424 Agilent chips hybridized in this study. Slides were then hybridized with labeled RNA, washed, scanned with an Agilent G2505A scanner. Features were extracted with background correction using Agilent G2566AA Feature Extraction Software Version A.6.1.1.Further details of the experimental protocols are given in Appendix A.

Statistical Analysis

The AfCS (Alliance for Cellular Signaling) murine B cell single ligand screen microarray dataset was used in the present study. (available at http://www.signaling-gateway.org/data/micro/cgi-bin/micro.cgi?expt=bref).

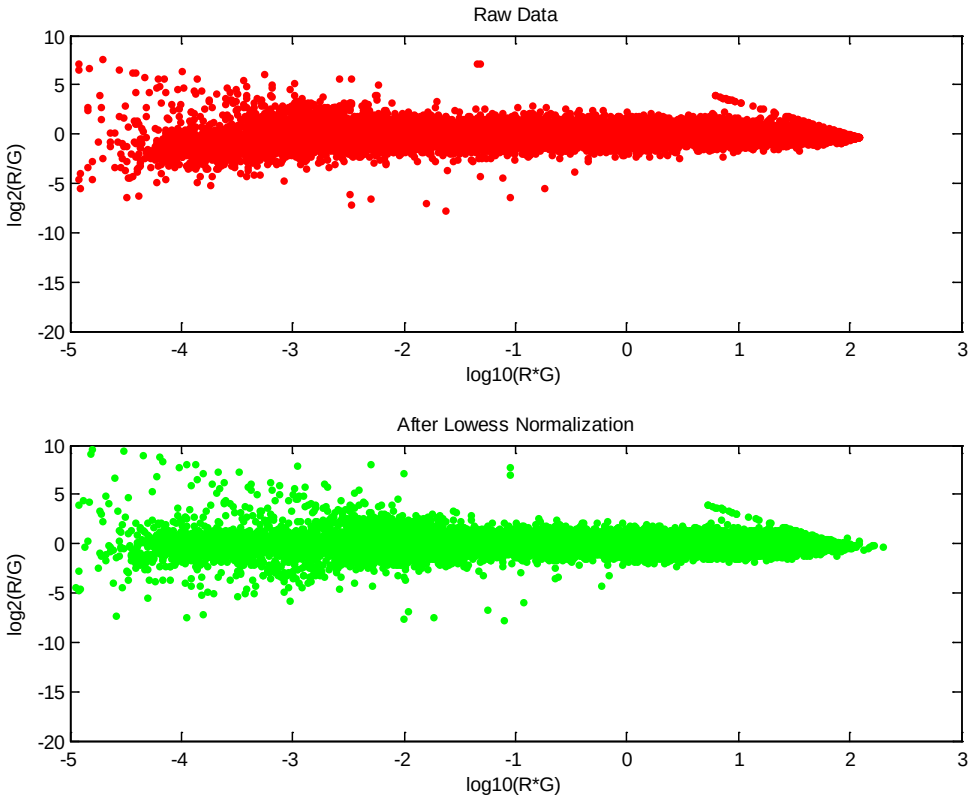


Figure 8 - Effects of Lowess Normalization on our data

The MatLab® Bioinformatics toolbox was used to process the data. The background corrected intensity values were used for each chip Probes showing negative values in background corrected intensities were replaced with a very low positive value (10, a value that was 500 times below the mean intensity of all chips). The assignment of a positive value was necessary to take the logarithm of

the data. All probes were also divided by the mean intensity of each chip in order to properly scale the intensity of all chips.

Lowess normalization was performed as a method of chip-to-chip normalization to allow for adequate analysis between chips. In this method, it is postulated that systematic dependence of $\log_2$(ratio) on intensity occurs, and must be corrected for in chips. (Quackenbush, 2002) Our data exhibited this dependence as well, illustrated in Figure 7. After the normalization, the replicate chips were averaged. To remove the outliers each replicated probe was subjected to an outlier test. The outlier test is as described:

1. All replicate chips were used for mean and standard deviation (SD) calculation.
2. Probes were selected in the range of mean ± 1.2 SD for the calculation of a new mean and SD
3. Possible outliers were tested in the range of the new mean ± 2 SD.
4. Probes found to be out of this range were discarded.

Fold change was calculated as ligand treated over control (untreated) samples for each probe on the chip. Known translation factors were next selected by Entrez GeneID (shown in Table 2), and multiple probes were averaged using the same outlier algorithm described above. Factors that did not meet a fold-change criteria of <0.875x or >1.25x control, for at least 25% of the treatment conditions were discarded. This allowed an examination of factors that showed a specific response to a particular ligand. Factors whose average

intensity + 2SD for all chips was found to be less than 20% of the mean intensity for all chips were also discarded, as these values were always close to the noise level of the chips. These cases can represent factors that are not expressed in B-cell or technically poor probes.

Hierarchical clustering was used to visualize the raw data, fold change, as well as to classify factors and experiments based on profile similarities. (Eisen *et al*, 1998) In our initial attempt, we used correlation as the distance metric. However, this clustered factors together that were broadly different in terms of up- vs. down-regulation. Thus, we resorted to using a combination of correlation and Euclidean distance as our metric in the cluster algorithm:

1. For the correlation component, we followed the standard formula $r_{xy} = \frac{\sum(x_i - \bar{x})}{(n-1)s_x s_y}$,
2. Then scaled it as $r'_{xy} = \frac{2}{1+r_{xy}}$, in order to provide a better scale to compare with Euclidean distances.
3. For the Euclidean component, we followed the standard formula $E_{xy} = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$,
4. $E'_{xy} = (E_{xy})^3$, was used to give less weightage when the distance is smaller than mean and vice versa when the distance is larger than mean. It was not enough to leave the standard Euclidean distances, as they were not great enough to make a noticeable effect over correlation until a power was added.

5. Both r'_{xy} and E'_{xy}were divided by their mean to give r''_{xy} and E''_{xy} in order to give equal weightage
6. Finally, r''_{xy} and E''_{xy} were averaged together to give a final distance metric, with non-linear weightage of correlation and Euclidean distance.

This distance metric was implemented as a MatLab® script, and fed into the built-in clustergram script.

In order to visualize temporal gene expression changes in response to ligand treatment, the average fold-change for each initiation factor for each replicate experiment was taken and plotted over time. The relevant inflammatory ligands were selected upon meeting a fold-change criteria of <0.67x control or >1.5x control for at least one time point.

Results and Discussion

Section A: Expression Profiles of Eukaryotic Initiation Factor 2 Subunits and α-kinases in Murine B Cells Following Treatment with Ligands

Global expression profiles of Initiation factors

Figure 9 shows a global overview of expression values for all eukaryotic initiation factors studied by hierarchical clustering employing the described distance metric (see Material and Methods). As indicated in the legend the data shown here represents the base-2 logarithm of fold change relative to untreated control samples. To highlight areas of interest regions indicating up- and down-regulation are outlined in yellow and grey, respectively.

Hierarchical clustering of translation initiation factors at 30 minutes of treatment with each of the 33 ligands was examined (Figure 10). Interestingly, the pattern of expression shows distinct areas of up-regulation (blue box) and down-regulation (green box) with the range of ligands used. A similar analysis of translation initiation factors at 60 minutes was also carried out (Figure 11) and the pattern observed at the earlier time point (30 min) was found to persist. However, individual initiation factors were up- and down-regulated at the later time point indicating a time as well as ligand dependence.

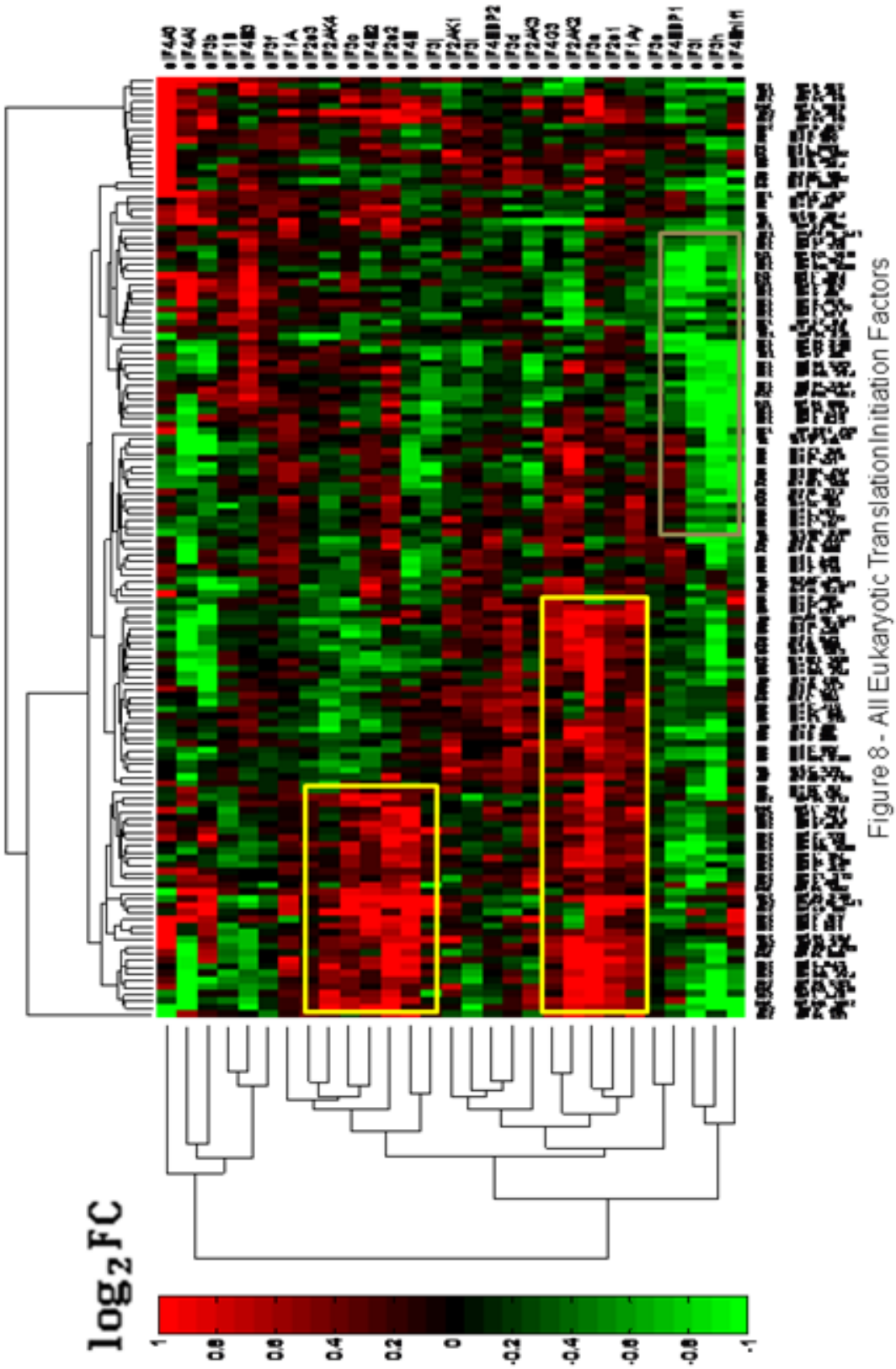


Figure 8 - All Eukaryotic Translation Initiation Factors

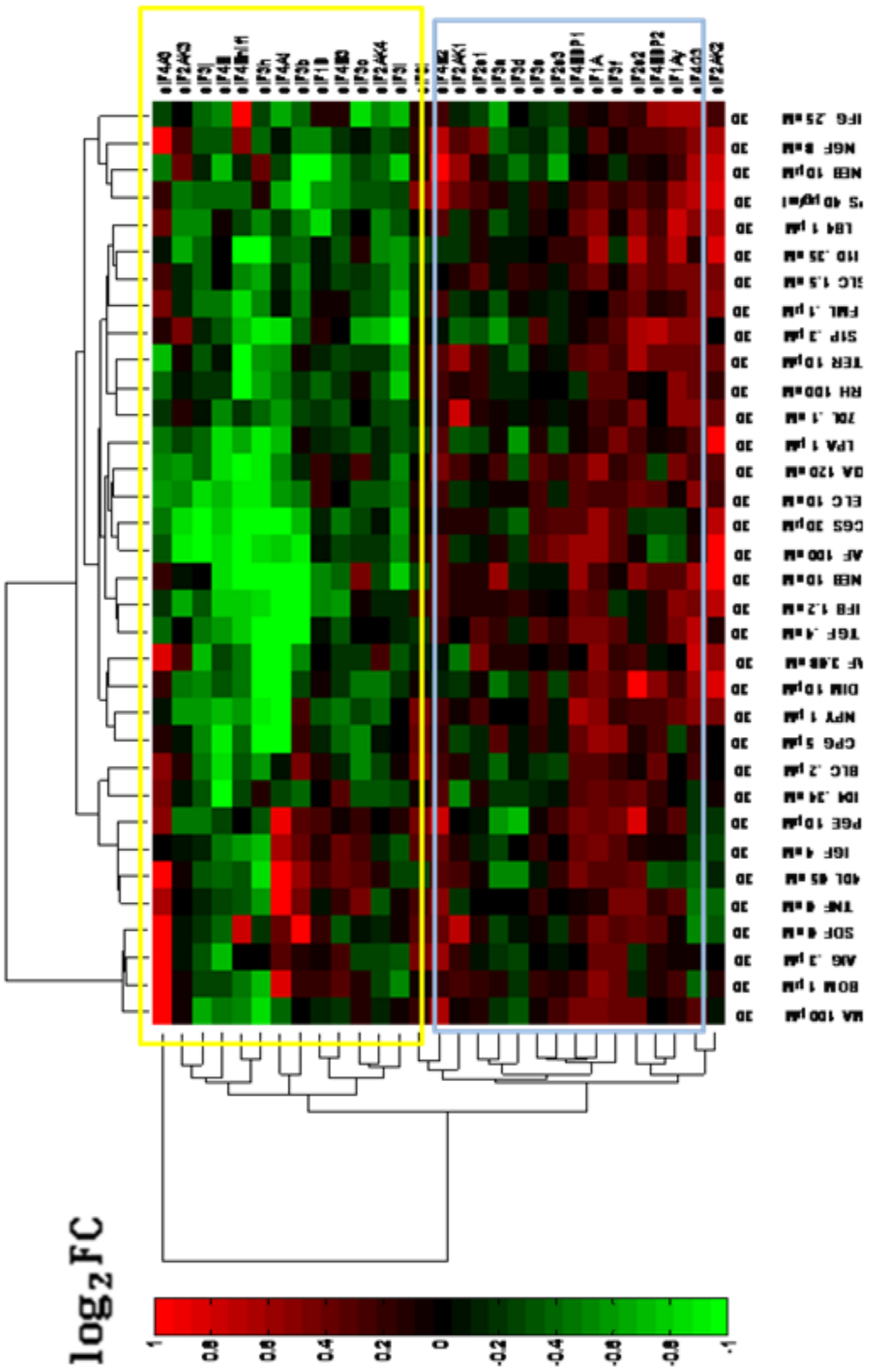


Figure 10 - Eukaryotic Translation Initiation Factors at 30 minutes

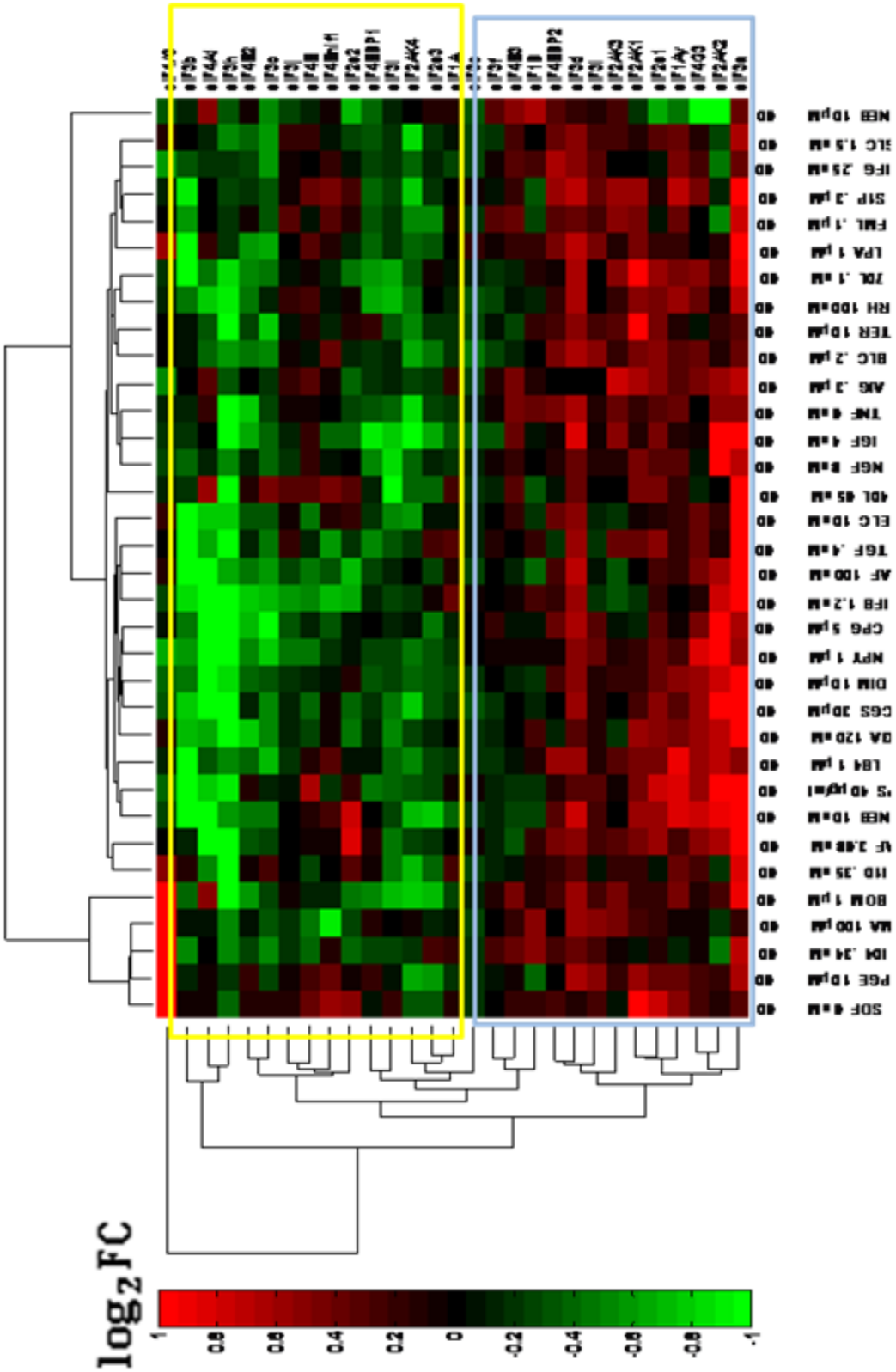


Figure 11 – Eukaryotic Translation Initiation Factors at 60 minutes

eIF2 Subunits and α-kinases 1-4

eIF2 is an initiation factor involved in the rate-limiting step in mRNA translation. It binds GTP and Met-tRNA and transfers Met-tRNA to the 40S ribosomal subunit. At the final step of the initiation process, GTP bound to eIF2 is hydrolyzed to GDP and the eIF2: GDP complex is released from the ribosome. The eIF2B factor is involved in GTP exchange and is a prerequisite to binding Met-tRNA. The eIF2s1 is phosphorylated at Ser-51 by four known alpha kinases that are regulated by different stimuli. This converts eIF2 from a substrate of eIF2B into a competitive inhibitor effectively preventing formation of the ternary complex and thereby inhibiting global protein synthesis. Phosphorylation of eIF2s1 occurs under a variety of conditions including viral infection, apoptosis, nutrient deprivation, heme-deprivation, and certain stresses.

A comparison of the three subunits of eIF2, previously known as the α, β and γ subunits and now referred to as eIFs1, eIFs2, and eIFs3 (Figure 12), shows highly discordant regulation. The GTP binding factor eIF2s3 does not indicate much change from the expression values seen for control samples, indicating that the expression of this subunit is poorly responsive to any of the ligands tested. In contrast to the poor response of eIF2s3 to the ligands screened, it was observed that eIF2s2 is markedly up-regulated at 240 min. eIF2s1, the key regulatory subunit in eIF2 whose phosphorylation can block translation, is mostly up-regulated across all ligands especially at 120 and 240 min of ligand treatment.

There are 4 known eIF2 α-kinases (eIF2AK1, -2, -3 and -4) that are induced in response to different stimuli (Figure 12) and these kinases also show discordant regulation. For example, eIF2AK-2 shows strong induction in a group of ligands at 60 min (CPG, NPY, NGF, CGS, IGF, LPS, and LB4), and strong down-regulation with another group of ligands at 120 min (SDF, LB4, NGF, IFG, TGF, SLC, TNF, BOM, TER, and IGF) (white box). As indicated earlier, eIF2s1 is phosphorylated by one or more of these kinases, leading to the inactivation of the ternary complex and thereby translation. For one set of ligands at 240 min (NEB, M3A, TER, SDF, LPS, BAF, TGF, NPY, 2MA, DIM, CGS, LB4, ELC, PAF, IFB, and CPG), eIF2 α-kinase 4 shows positive correlation with eIF2s1, indicating that expression of this gene is likely to influence phosphorylation of eIF2s1. Interestingly, anti-correlation (grey boxes) was observed with another group of ligands at 60 min (NGF, CGS, IGF, LPS, LB4, DIM, NGF, NEB, SDF, 40L, ELC, I10, FML, S1P, TER, 70L, AIG, TGF, GRH, LPA, PGE, BLC, TNF, and BOM) and similar patterns were observed with eIF2 α-kinase 3 where certain ligands showed opposing effects while others showed coordinate expression of eIF2s1 and eIF2AK-3 (Figure 12, yellow box).

The expression profiles of all three eIF2 subunits and the four alpha kinases were examined for a group of inflammatory ligands that include AIG, LPA, LB4, PAF, PGE, DIM, I10, IFG, IFB, LPS, CPG, TNF, 4OL, BAF and IO4. Since eIF2s1 is phosphorylated by the alpha kinases it was interesting to examine its response to each of these inflammatory ligands. Of the 15 ligands in

the inflammatory group it was observed that eIF2s1 gene expression was only changed with 6 ligands (40L, BAF, DIM, I10, IFG and LPS). Further, α kinase 1 was only effected by BAF whereas α kinase 2 by most inflammatory ligands. The expression of α kinase 3 was altered for a subset of ligands namely 40L, AIG, LB4 and LPS. The expression of α kinase 4 was unaffected by most inflammatory ligands but altered following treatment with BAF, I10 and IFG.

Opposing effects between eIF2s1 (Figure 13) and kinase expression were observed for some ligands. For instance, α kinase 2 shows an opposing effect with BAF and I10 treatment (Figure 14) whereas with α kinase 3 this effect was seen with 40L and LPS treatment (Figure 15). Again, a similar opposing effect was observed for α kinase 4 for 3 ligands (BAF, I10 and IFG) at the earlier time points of treatment (Figure 16).

Similar expression profiles were observed between eIF2s1 and kinase expression with a select subset of inflammatory ligands. In the case of α kinase 2 treatments with DIM, IFG and LPS showed similar trends. For α kinase 3 similar expression patterns were observed for the earlier time points of treatment with LPS and 40L. In the case of α kinase 4, the fold-change was similar at the later time points with BAF, I10 and IFG treatment.

In this study it was interesting to investigate whether the gene expression of all 3 eIF2 subunits were coordinately regulated following exposure of murine B cells to the different ligand groups. Our analysis indicates that eIF2 subunit 3 was poorly responsive to any of the ligand treatments. However, eIF2s1, the key

regulatory subunit was induced to different extents at an early time point (60 min) and some ligands (LPS and 40L) showed decreased expression at the later time point (240 min) whereas certain ligands (BAF, DIM, I10 and IFG) induce expression at 240 min. Since up-regulation of the α kinases is an indicator of eIF2s1 phosphorylation, its up-regulation will suggest that global translation in B cells exposed to certain ligands (LPS, DIM, IFG, 40L, and BAF) will be impaired at certain time points. On the other hand certain ligands (LPS and AIG) show marked down-regulation of alpha kinase 2 at 120 min indicating that eIF2s1 is most likely not phosphorylated thus enabling translation to proceed.

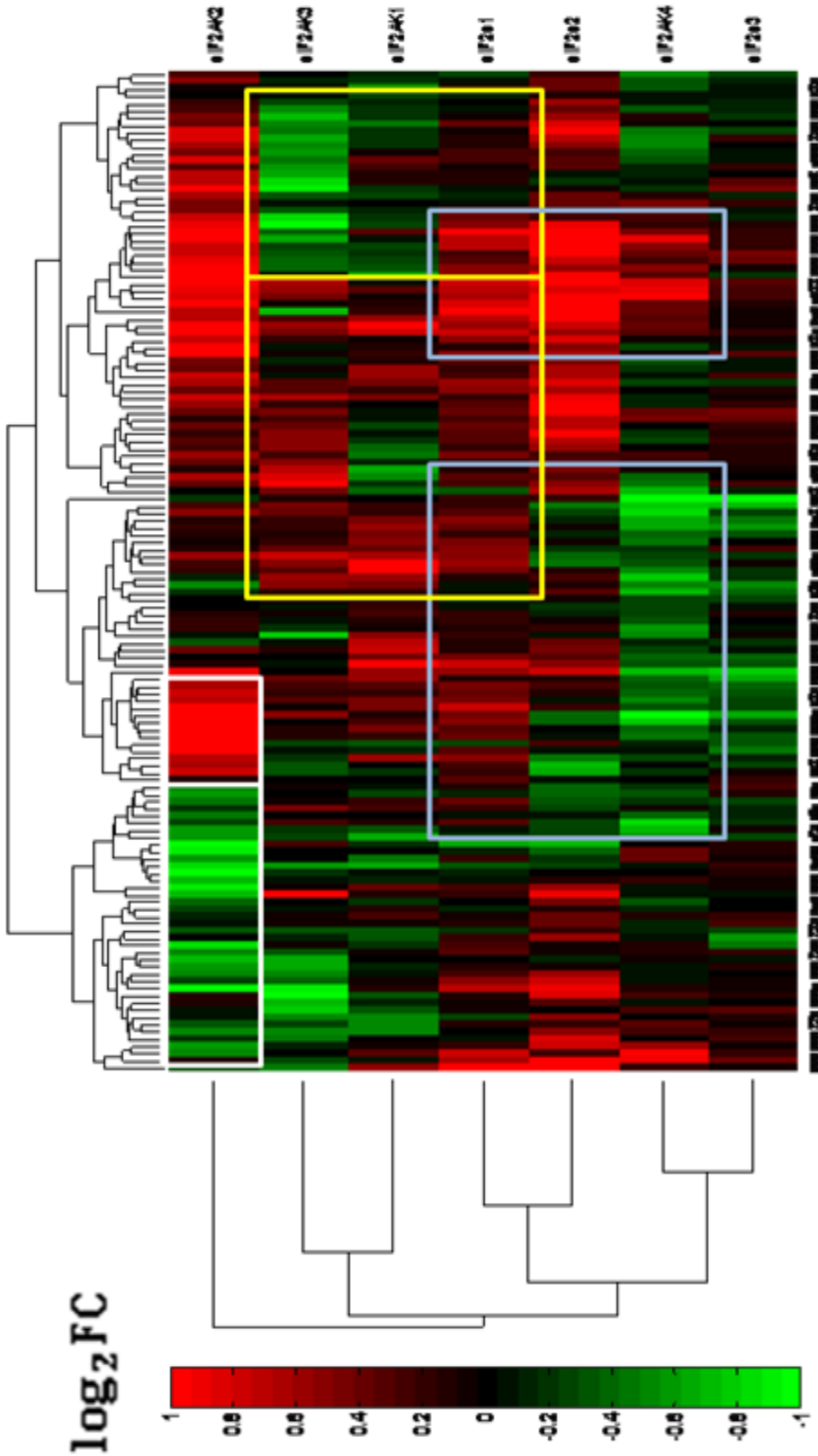


Figure 12 – Eukaryotic Initiation Factor 2: Discordant Regulation of subunits and alpha kinases

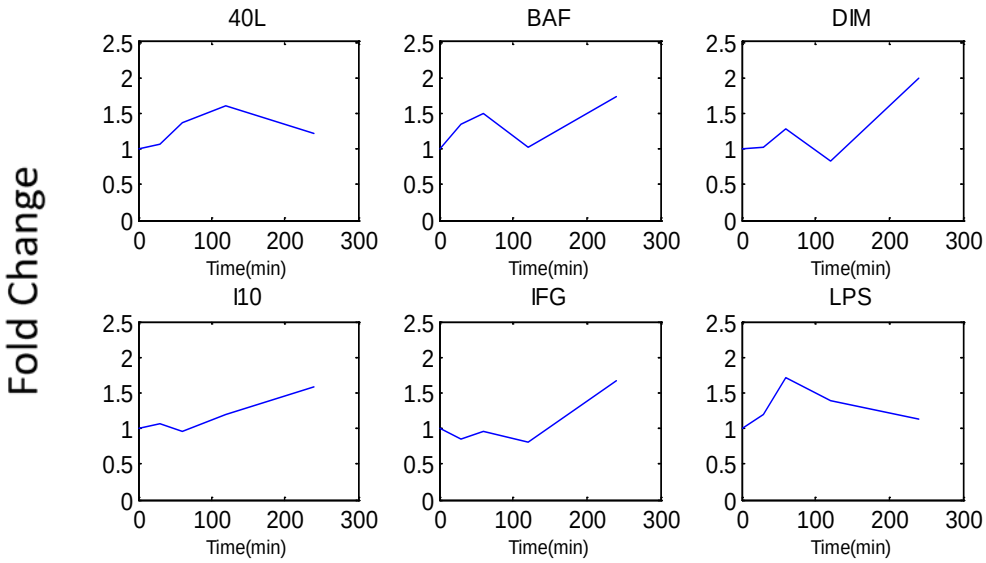


Figure 13 - Gene Expression Profiles of eIF2 Subunit 1 following treatment of B cells with selected Inflammatory Ligands.

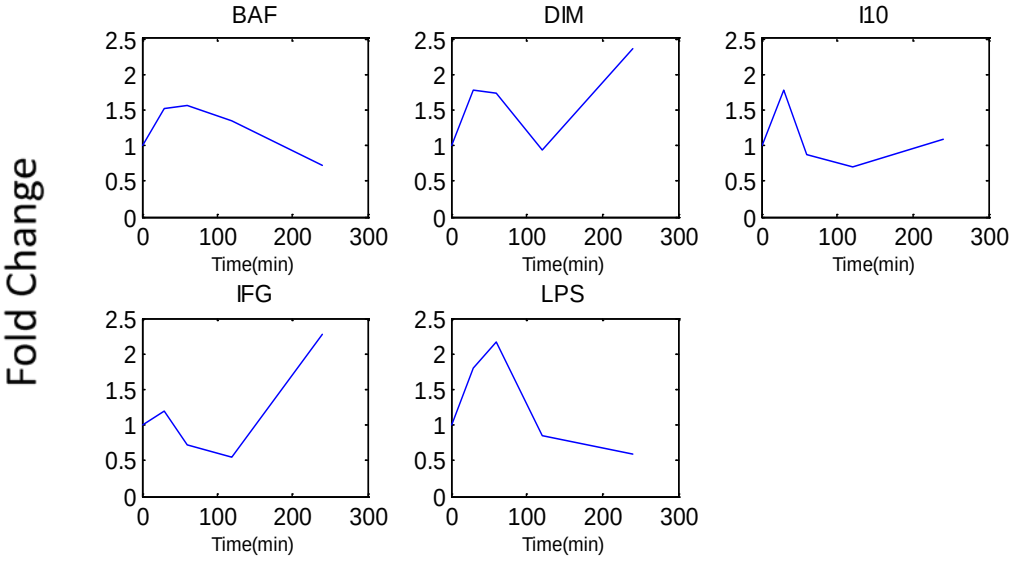


Figure 14 - Gene Expression Profiles of eIF2 AK2 following treatment of B cells with selected Inflammatory Ligands.

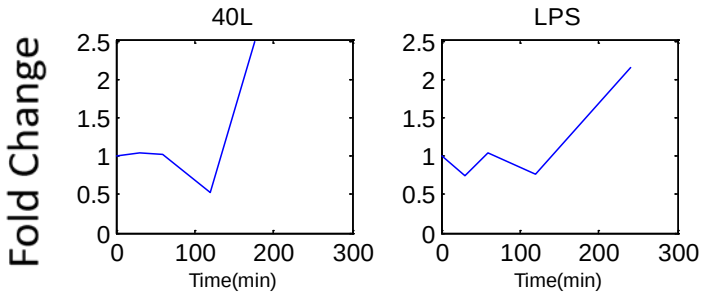


Figure 15 - Gene Expression Profiles of eIF2 AK3 following treatment of B cells with selected Inflammatory Ligands.

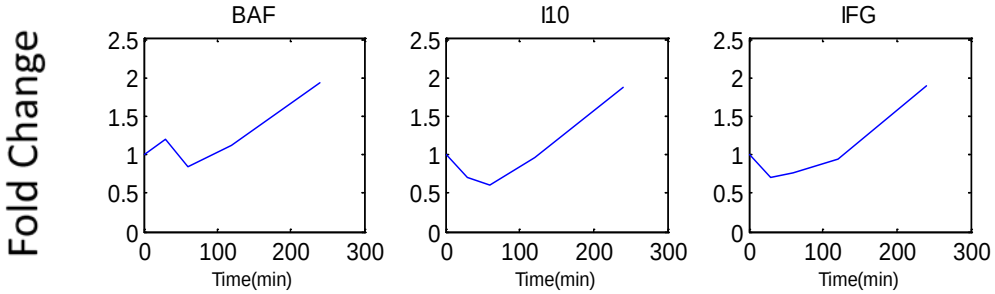


Figure 16 - Gene Expression Profiles of eIF2 AK4 following treatment of B cells with selected Inflammatory Ligands.

Section B: Expression Profiles of Eukaryotic Initiation Factor 3 Subunits in Murine B Cells Following Treatment with Ligands

Much of our understanding of eIF3 has arisen from studies undertaken in the last decade. Most of the reactions in the initiation pathway are stimulated by eIF3 interactions including the binding to the ternary complex. It is now known that eIF3 interacts with other initiation factors to facilitate the translation process (Hinnebusch, 2006). The eIF3 is a large multi-function complex that contains over 12 non-identical subunits (Table 2). Recent studies in budding yeast have implicated eIF3a, b, c, g and I, as a conserved 'core' complex that is of vital importance to translation.

It can be seen in Figure 17 that all eIF3 subunits are regulated discordantly with ligand treatments at each of the time points. Interestingly, genes for the various subunits were both up- and down-regulated with the same treatment at a given time point. The eIF3a subunit was one of the few subunit genes that were markedly changed both up-regulated with treatment at 60 and 240 min and down-regulated at 30 min (Figure 17, grey box).

The temporal expression profiles showed similar trends for eIF3a (Figure 18) with most inflammatory ligands with the exception of TNF, which showed no change. In contrast the expression of eIF3h and eIF3l genes was markedly down-regulated with all ligands and at all time points studied (Figure 17, yellow box). In addition, the expression profiles for eIF3a were similar with the other ligand groups tested. With the exception of I04, IFG, and PGE which did not influence eIF3b expression, most other inflammatory ligands induced the

expression of this subunit. The inflammatory ligands stimulating the expression of the eIF3c subunit were quite different in that they induced gene expression changes for all ligands with the exception of DIM, and LPS. Further, it was observed that eIF3e, eIF3f, and eIF3i showed minimal up- or down-regulation following treatment with the present set of inflammatory ligands.

In the present study, employing 424 Agilent chips and 33 single ligands, it was of interest to examine whether the different eIF3 subunits (a-l) in B cells were coordinately regulated with each ligand treatment and further, whether similar patterns of expression occurred for the different ligand classes tested. As mentioned above the eIF3a, b, c, g and I constitute the 'core' eIF3 machinery based on the studies in budding yeast (Hinnebusch, 2006). Interestingly, the present studies show that eIF3a, eIF3b, eIF3c, and eIF3l are strongly influenced by most inflammatory ligands (eIF3g was not present on our chips). Thus, it appears translation is markedly influenced by inflammatory ligands, based on the assumption that this core complex is necessary for protein synthesis.

Surprisingly, these studies show that eIF3 subunits are not coordinately expressed in B cells with any of the ligand treatments and even the 'core' subunits of eIF3 were not coordinately expressed. Further, eIF3a was up-regulated with a subset of ligands whereas eIF3h and eIF3l were markedly down-regulated with most ligands. The significant up-regulation of eIF3a (previously known as p170) could be related to its dual role as a mediator of translation of a subset of mRNA localized specific cellular compartments.

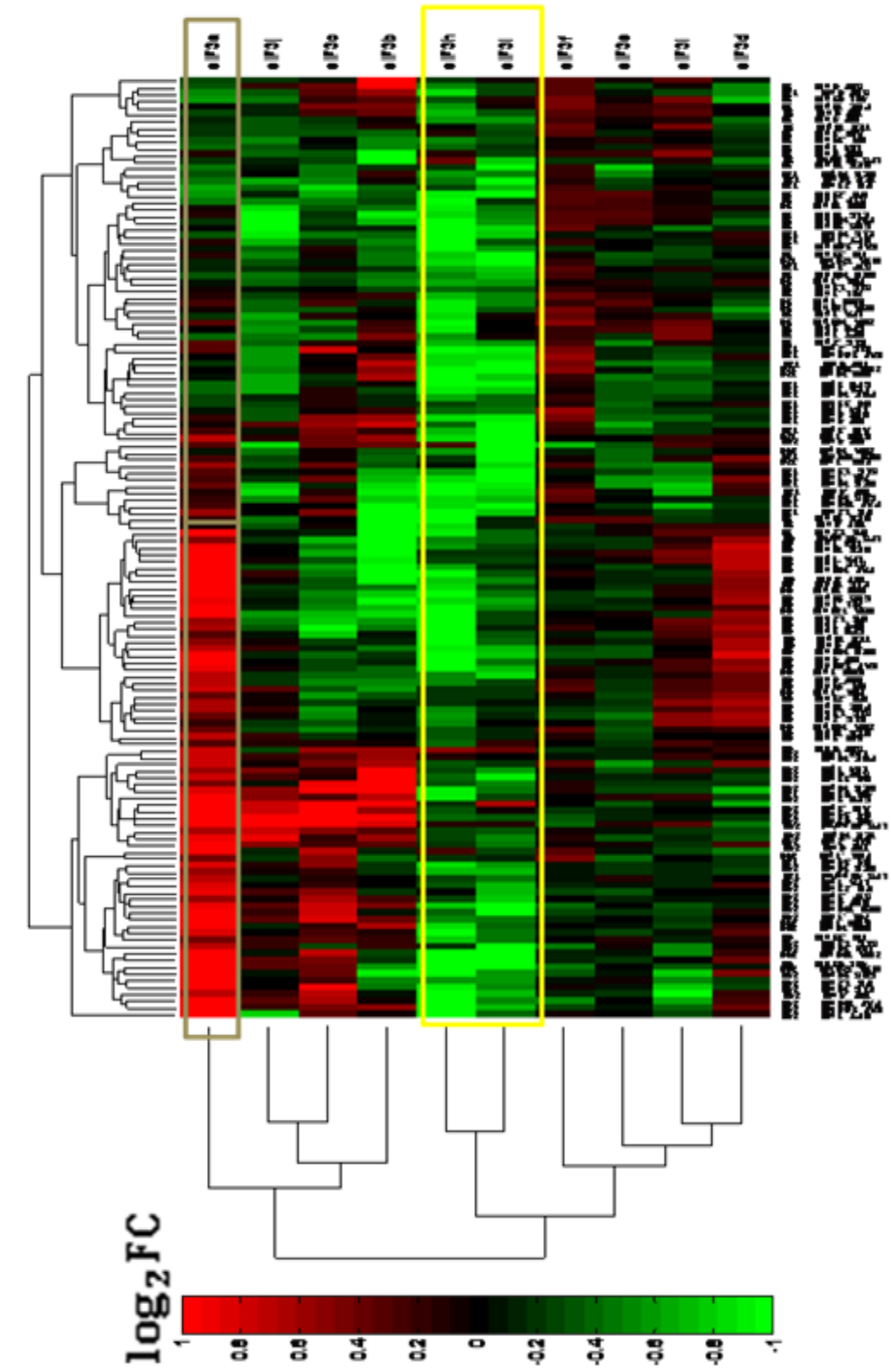


Figure 17 – Eukaryotic Initiation Factor 3: Discordant Regulation of Ten subunits

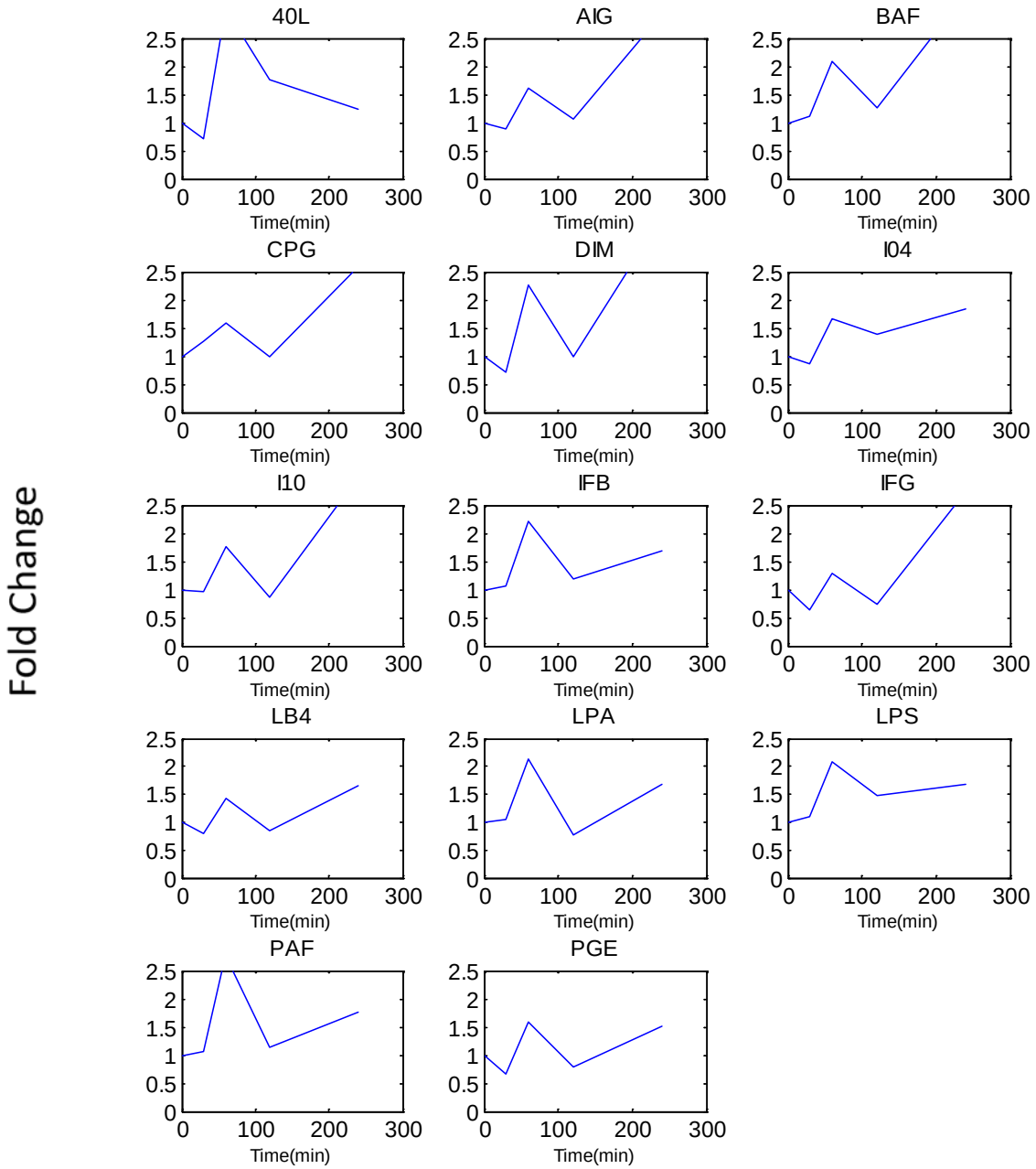


Figure 18 - Gene Expression Profiles of eIF3a following treatment of B cells with selected Inflammatory Ligands.

Section C: Expression Profiles of Eukaryotic Initiation Factor 4E Isoforms and 4E-Binding Proteins in Murine B Cells Following Treatment with Ligands

The eukaryotic initiation factor 4F complex, comprising 3 subunits (eIF4A, eIF4E and eIF4G) is unique because each subunit has multiple isoforms that are expressed in a tissue dependent manner. eIF4A is responsible for performing an RNA helicase function in order to assist in scanning for the start codon in mRNA. Studies have shown that free eIF4A (unbound to eIF4G in the eIF4F complex) still contains RNA helicase activity. Free eIF4A on its own however, without the eIF4F complex, demonstrates far less RNA helicase activity. Thus, in conjunction with the high eIF4A presence in the cell (three to six-fold more than the other initiation factors), it can be inferred that the cellular machinery utilizes free eIF4A for increasing translation efficiency. (Gingras *et al*, 1999)

Two isoforms of eIF4A, eIF4A1 and eIF4A3, are expressed in murine B cells. Interestingly, eIF4A2 is not seen to be expressed in the present study. (Figure 19)The eIF4A1 isoform is significantly down-regulated with one group of ligands (CGS, BAF, DIM, NPY, NEB, PAF, IFB, and CPG), while eIF4A3 is markedly up-regulated for another group of ligands mostly at 30 and 60 min (NGF, BOM, 2MA, SDF, S1P, I04, PGE, and AIG) (Figure 19, white boxes) indicating that the pattern of regulation for these isoforms was markedly different.

eIF4G has two known functions: to act as a bridge between the ribosome and mRNA, as well as to circularize the mRNA molecule via interaction with

Poly(A)-Binding Protein (PABP). (Gingras *et al*, 1999) It was interesting that only eIF4G isoform 3 was expressed in murine B cells with any of the ligand treatments and no expression signals were present for the other isoforms. In the case of eIF4E and eIF4E isoform 2 it was observed that these isoforms show coordinate regulation with a set of ligands at 240 min (TER, PGE, LB4, FML, ELC, 2MA, 40L, NGF, LPS, AIG, NEB), while for a different group of ligands, they show opposing regulation (NEB, 70L, LPS, CPG, NPY, IFB, TGF, TER, ELC, LPA, M3A, BAF, CGS, GRH, and FML). In addition, opposing regulatory responses were observed for eIF4E isoform 1 and 2 versus eIF4E isoform 3 with a set of ligands at 240 min (LB4, TGF, NPY, CPG, IFB, M3A, CGS, and PAF) (Figure 19, yellow boxes).

Upon further examination with the group of inflammatory ligands, we noticed that eIF4A1 showed altered regulation for 40L, AIG, BAF, IFG, LPS, PGE and TNF. (data not shown) eIF4A3 showed altered regulation for 40L, AIG, BAF, I04, I10, IFB, LPS, PGE and TNF. (data not shown)

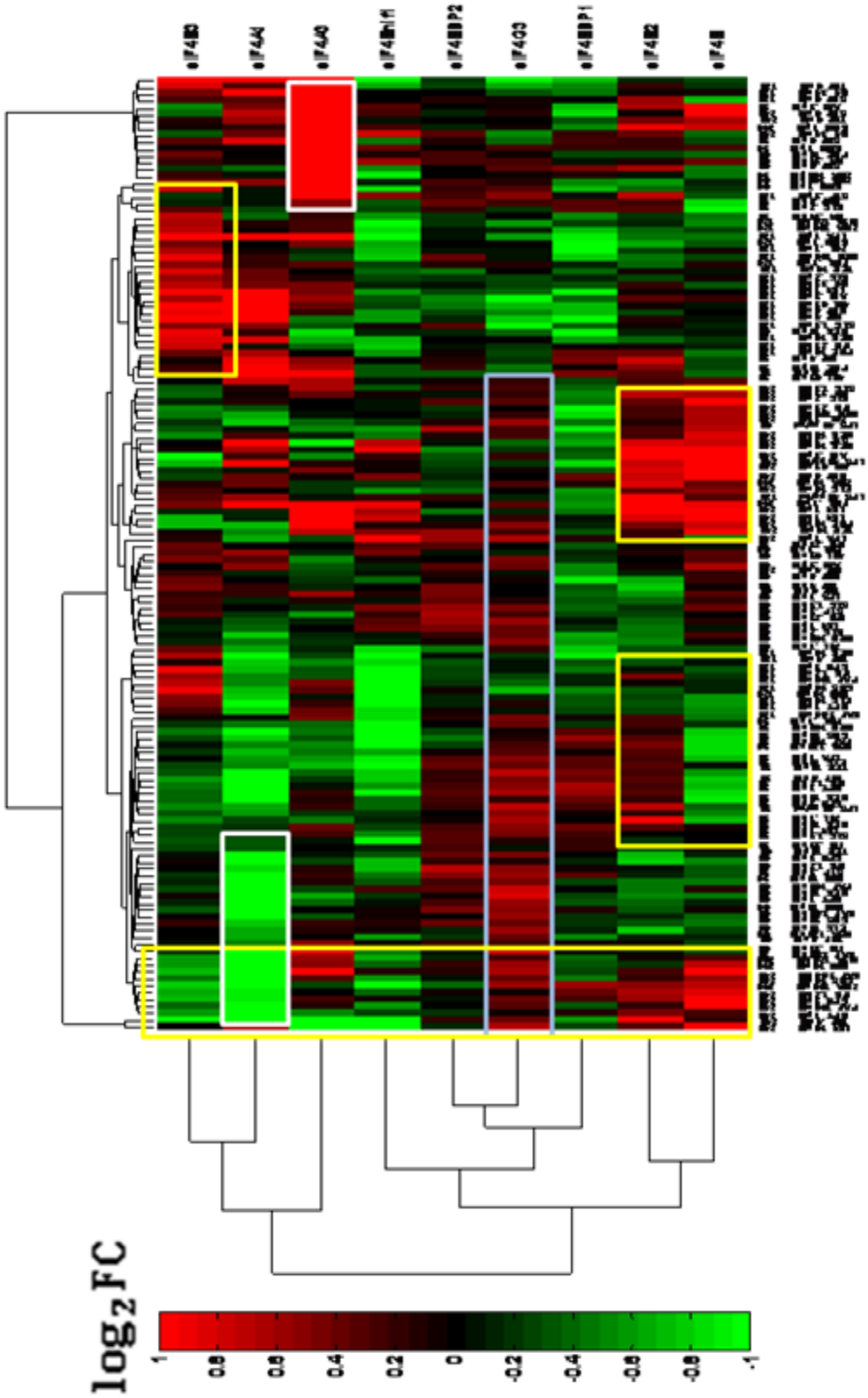


Figure 19 - Eukaryotic Initiation Factor 4: Discordant Regulation of Subunit Isoforms

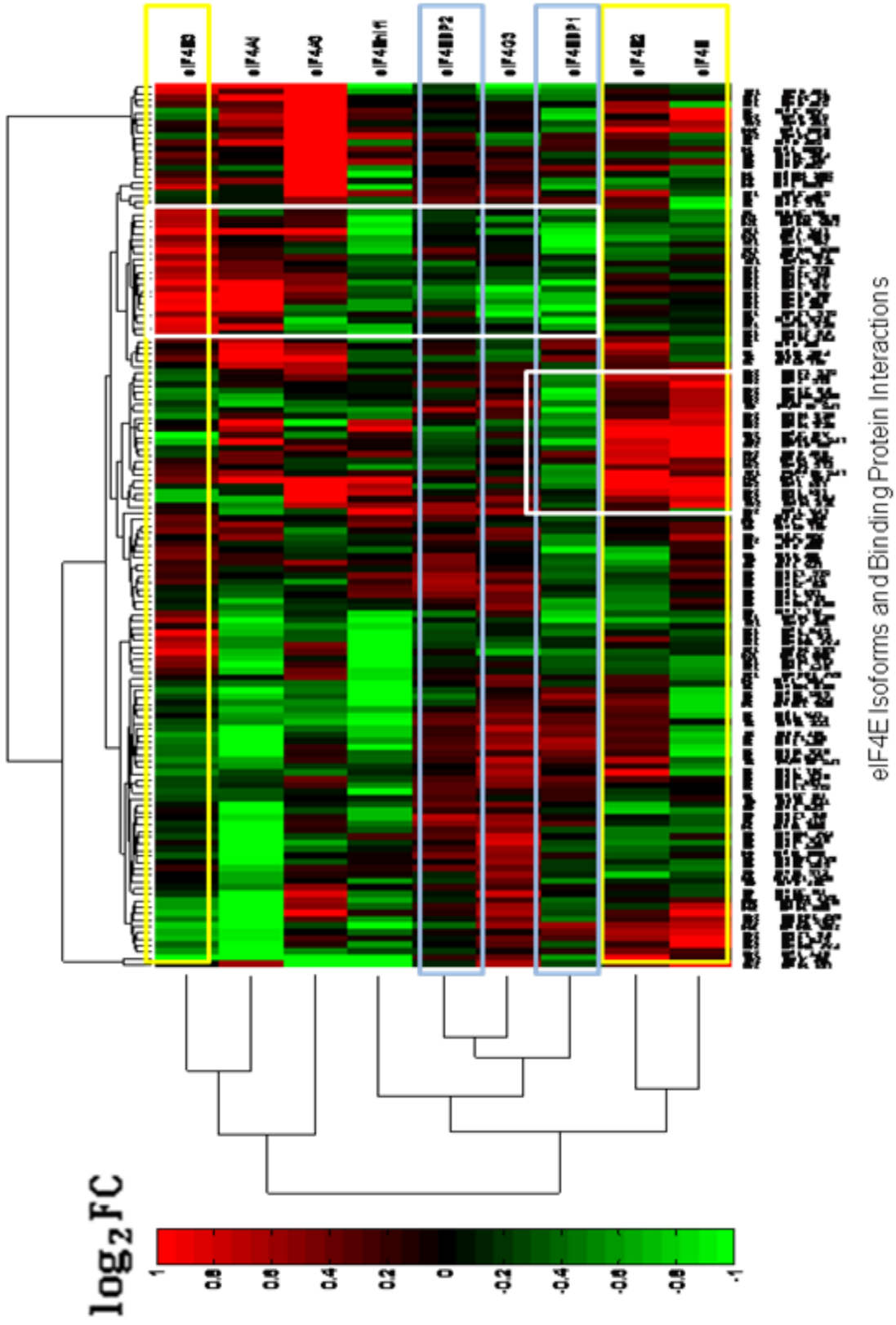
eIF4E functions to recognize the 5’ cap of mRNA, allowing cap-dependent translation to occur. Another fundamental process, whereby translation can be inhibited, is through competitive binding of eIF4E- binding proteins with the 5’ cap of mRNA. By this process, a cell shuts down translation as the eIF4F complex cannot bind to mRNA. (Gingras *et al*, 1999)

The eIF4E-Binding proteins and their interactions with eIF4E isoforms is shown in Figure 20. For convenience the expression values for eIF4E isoforms are boxed in yellow, while the eIF4E-BP1/2 expression values are boxed in grey. It was observed that eIF4E-BP1 is mostly unaffected by ligand treatment, while most ligands down-regulated the expression of eIF4E-BP2. Opposing regulatory effects can be seen between eIF4E-BP2 and all three eIF4E isoforms in a ligand dependent manner. For example, in a group of ligands at 240 min (TER, PGE, LB4, FML, ELC, 2MA, NGF, 40L, LPS, and AIG), eIF4E-BP2 is markedly down-regulated, while eIF4E and eIF4E2 gene expression is markedly up-regulated. In another case, eIF4E-BP2 is down-regulated, while eIF4E3 is up-regulated for a group of ligands at 120 min (NEB, SLC, IGF, NGF, 40L, AIG, LB4, BLC, FML, GRH, 70L, BOM and LPA). In yet another example, eIF4E-BP2 is up-regulated for a group of ligands at 30 min (I10, SLC, LB4, NEB, LPS, CPG, NPY, IFB, TGF, TER, and ELC), while eIF4E and eIF4E3 gene expression is down-regulated. These opposing regulatory effects are outlined in white.

When the eIF4E isoforms and eIF4E-BPs were examined for the inflammatory ligands, it was seen most inflammatory ligands altered gene

expression for eIF4E (Figure 21) and eIF4E3 (Figure 23) isoforms, while eIF4E2 (Figure 22) was only altered for 7 ligands (40L, AIG, BAF, I10, IFG, LPS, and PGE). It was also seen that eIF4EBP1 was only altered slightly for PAF when studied for the group of inflammatory ligands. Anti-correlation was observed between eIF4E and eIF4E-BP2 (Figure 24) at an early time point (30 min). On the other hand, this effect is seen at a later time point (240 min) between eIF4E3 and eIF4E-BP2

Interestingly, the various isoforms of each eIF4F subunit showed marked differences in gene expression upon treatment to the inflammatory ligands. eIF4G only expressed isoform 3, consistent with tissue-specific isoform expression. eIF4E and eIF4A subunits expressed multiple isoforms at the gene expression level upon single ligand treatment. Further, eIF4E-BP2 was the only binding protein that was markedly altered by ligand treatment, with eIF4E-BP1 showing minimal gene expression changes. Thus, the cellular machinery in large part, determines the nature of translation in B cells upon external stimulation.


eIF4E Isoforms and Binding Protein Interactions
eIF4E
eIF4G2
eIF4EBP1
eIF4G3
eIF4EBP2
eIF4ENIF1
eIF4A2
eIF4A1
eIF4G1
log2FC
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

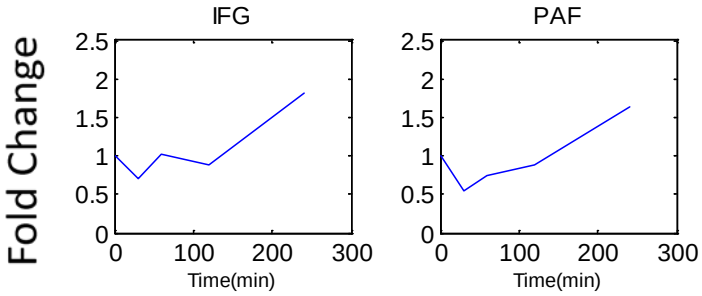


Figure 22 – Gene Expression Profiles of eIF4E following treatment of B cells with selected Inflammatory Ligands.

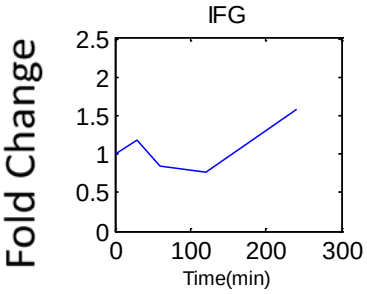


Figure 23 – Gene Expression Profiles of eIF4E2 following treatment of B cells with selected Inflammatory Ligands.

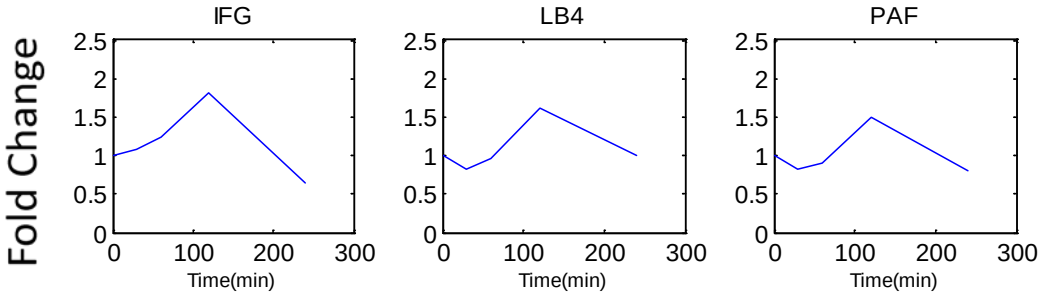


Figure 24 - Gene Expression Profiles of eIF4E3 following treatment of B cells with selected Inflammatory Ligands.

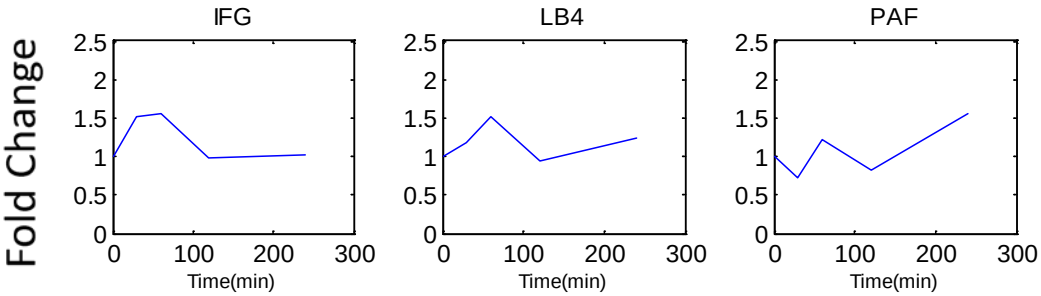


Figure 25 - Gene Expression Profiles of eIF4E-BP2 following treatment of B cells with selected Inflammatory Ligands.

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Appendix A – Experimental Protocols

Isolation of B-lymphocyte RNA

Freshly isolated splenic B lymphocytes were suspended at $16.7\ x\ 10^6\ {}^{cells}/_{ml}$ in Supplemented Iscove's Modified Dulbecco's Medium (SIMDM) and 1.08 ml was distributed into individual wells of 12-well tissue culture plates as needed. These were incubated at 37 °C in air with 5% CO_2 for 1 hr, and transferred to an environmental chamber with air at 37 °C. 0.12 ml ligand was added (10X final concentration in SIMDM) to appropriate vehicles to begin treatments. Plates were placed on shaker and rotated for 30 sec. Cells were then incubated once again at 37 °C in air with 5% CO_2 for desired times. Cells were then transferred from individual wells to 15-ml barcoded conical tubes at the end of treatment and centrifuged at room temperature for 5 min at 400 x g. Supernatants were then removed with sterile 5-ml plastic pipettes. 1 ml of ice-cold TriPure was added to each of the wells and mixed with the cells to lyse them. The lysate was transferred to 1-ml sterile plastic pipettes to the corresponding conical tubes. A further 2 mL of TriPure was added to each conical tube to a final volume of 3 ml. Samples were mixed by pipetting, and then transferred to a 13-ml barcoded polypropylene tube, which was stored at -80 □C until ready for RNA isolation. Samples were then transferred to room temperature, and 0.6 ml of chloroform was added immediately after thawing, and tubes were shaken for 30 sec. The mixture was then incubated at room temperature for 5 min. Samples were centrifuged at 4 °C for 15 min at 12000 x g. The colorless upper aqueous phase

was transferred to a new 13-ml barcoded polypropylene tube. The extraction was repeated by adding 2 ml chloroform to the transferred tube. 1.5 ml Isopropanol was then added to the final, isolated aqueous phases, and mixed by inverting. The sample was incubated at room temperature for 5 min, and centrifuged at 4 °C for 10 min at 12000 x g. All but 0.5 ml of the remaining supernatant was removed. The sample was vortexed to disperse the RNA pellet and transferred to a barcoded Eppendorf tube. Sample was centrifuged again for 5 min at 4 °C at 18000 x g in a microfuge. Supernatant was carefully removed and 1 ml RNase-free 70% ethanol was added and vortexed. Sample was centrifuged at 18000 x g for 5 min at 4 °C. Supernatant was carefully removed and air-dried for 30 min. 10 µl nuclease-free water was added, and sample was incubated on ice for 20 min, vortexing at 5 min interval. RNA concentration was determined by diluting 1:80 in Tris-EDTA buffer and reading A260 in a spectrophotometer. Total RNA integrity was determined by electrophoresis of 1 µg RNA on 1% agarose gel and staining with ethidium bromide for 18S and 28S ribosomal RNA. Samples were stored at -80 °C prior to microarray analysis.

Reverse Transcription, Labeling, and Microarray Hybridization

5 µg total RNA was added in nuclease-free water to a 1.5 ml RNase-free tube, along with 1 µl of 100 $^{\mathrm{pmol}}/_{\mu\mathrm{l}}$ T7-$(dT)_{24}$ primer, with the volume adjusted to 12 µl/reaction with nuclease-free water. Mixture was incubated at 70 °C for 10 min, centrifuged briefly, and placed on ice. 4 µl of 5X first strand cDNA buffer, 2 µl of

0.1 M DTT, 1 µl of 10 mM dNTP mix, and 1 µl of 200 U/µl SuperScript II RT was added to the mixture, mixed well by pipetting 4 to 5 times, incubated at 42 °C for 1 hr, centrifuged briefly and placed on ice. 30 µl of 5X second strand buffer, 3 µl of 10 mM dNTP mix, 1 µl of 10 U/µl E. coli DNA ligase, 4 µl of 10 U/µl E. coli DNA polymerase I, 1 µl of 2 U/µl RNase H and 91 µl of nuclease-free water was added to the reaction, and incubated at 16 µl °C for 2 hr. 2 µl of 5 U/µl T4 DNA polymerase was added and incubated at 16 °C for 5 min. 7.5 µl of total RNA digestion solution was added, incubated at 65 °C for 10 min, centrifuged briefly, and placed on ice. A Phase Lock Gel tube was prepared by centrifuging at 12000 x g for 30 sec. 162 µl of 25:24:1 phenol: chloroform: isoamyl alcohol was added to the synthesized cDNA and mixed well by pipetting for 4 to 5 times. cDNA mixture was transferred to the Phase Lock Gel tube and centrifuged at 12000 x g for 3 min. Upper aqueous phase was transferred to a fresh 1.5 ml RNase-free tube, and 75 µl of 7.5 M ammonium acetate was added, as well as 375 µl -20 °C 100% ethanol. Solution was mixed well by pipetting and centrifuged at 14000 x g for 5 min at room temperature. Supernatant was carefully removed, and then the pellet was washed with 0.5 ml -20 °C 100% ethanol, and centrifuged again at 14000 x g for 5 min at room temperature. Supernatant was again carefully removed, and pellet was air-dried for 10 min, and then resuspended in 16 µl of nuclease-free water, and stored at -20 °C RNA polymerase enzyme mix from Ambion MEGAscript T7 Kit was placed on ice. 10X reaction buffer and 4 ribonuclelotide solutions were vortexed until they were thawed, and then placed at room temperature. All reagents were centrifuged briefly. 4 µl each o f ATP

solution, CTP solution, GTP solution, UTP solution, 4 µl of 10X reaction buffer, 16 µl of cDNA template and 4 µl enzyme mix were mixed at room temperature, mixed well by pipetting gently 4 to 5 times, and incubated at 37 °C for 4 hr. 1 µl of 2 U/µl DNase I was added and mixed well by pipetting, and then incubated at 37 °C for 15 min. 60 µl of nuclease-free water was added to the MEGAscript reaction. Phase Lock Gel tube was prepared by centrifuging at 12000 x g for 30 sec. 100 µl 25:24:1 phenol: chloroform: isoamyl alcohol was added to the synthesized cRNA, and mixed well by pipetting 4 to 5 times. The mixture was then transferred to the Phase Lock Gel tube and centrifuged at 12000 x g for 3 min. The upper aqueous phase was transferred to a fresh 1.5 ml tube, sample volume was adjusted to 100 µl with nuclease-free water. 350 µl RLT buffer was added from RNeasy Mini kit and mixed well by pipetting 4 to 5 times. 250 µl of 100% ethanol was added and mixed well by pipetting. Sample was then applied to an RNeasy Mini spin column sitting in a 2-ml collection tube and centrifuged at 8000 x g for 15 sec. The column was transferred to a fresh collection tube, 500 µl RPE buffer was added and centrifuged at 8000 x g for 15 sec. Flow through was discarded and column was placed in the same collection tube, 500 µl of RPE buffer was added and centrifuged at maximum speed for 2 min to dry the column membrane. Column was transferred to a fresh 1.5 ml collection tube, 30 µl of nuclease-free water was pipetted directly to the column membrane and centrifuged at 8000 x g for 1 min to elute RNA. 0.5 µl eluted RNA was removed to a fresh tube and 49.5 µl nuclease free water was added. RNA concentration was determined by A260 (An OD of 1 = 40 µg/ml RNA).3 to 10 µg *in vitro*

antisense RNA target was mixed with 2 µl of 3 µg/ml random primers, volume was adjusted to 14 µl/reaction with nuclease-free water, and the mixture was incubated at 70 □C for 10 min. 6 µl 5X first strand cDNA buffer, 3 µl 0.1 M DTT, 0.6 µl 50X dNTP (low dTTP) mix, 1.4 µl nuclease-free water, 3 µl Cy5-dUTP (or Cy3-UTP), and 2 µl 200 U/µl SuperScript II RT was mixed well by pipetting, and incubated at 42 °C for 2 hr. 500 µl Tris-EDTA buffer at pH 8.0 was added and the mixture was transferred to a Microcon-30 filter. The filter was centrifuged at 10000 x g for 9 min at room temperature, and centrifuged further to a final volume of 10 to 20 µl. A fresh tube was placed on top of filter unit, inverted and centrifuged at 10000 x g for 2 min. Recovered Cy3 and Cy5 labeled targets were mixed in a Microcon-30 filter containing 500 µl Tris-EDTA, and centrifuged at 10000 x g for 9 min at room temperature, and centrifuged further to a final volume of 7.5 µl. A fresh tube was placed, inverted, and centrifuged at 10000 x g for 2 min. 7.5 µl target solution was transferred to a fresh tube, 2.5 µl deposition control target, 2.5 µl 1 µg/µl mouse Cot-1 DNA, 12.5 µl 2X deposition hybridization buffer were added, mixture was heated at 98 °C for 2 min, and centrifuged at 10000 x g for 2 min. 25 µl of fluorescently labeled target was pipetted onto the slide surface, 24 x 30 mm cover slip was placed on top of the slide carefully to remove bubbles. Slide was placed in a hybridization chamber, and submerged in a 60 °C water bath overnight. Hybridization chamber was then disassembled with array surface facing up. Slide was immersed in 100 ml wash solution A1 at 42 °C for 3 min in a Coplin jar to remove the cover slip, and wash solution was decanted.100 ml wash solution A1 was added, and slide was

incubated at room temperature for 3 min. Wash solution was decanted, and 100 ml wash solution A2 was incubated at room temp for 22 min. Slide wash with A2 solution was repeated, and then final solution was decanted, and slide was centrifuged at 110 x g for 5 min to dry.