STUDY OF 3′-UNTRANSLATED REGION OF INDUCIBLE NITRIC OXIDE SYNTHASE AND IDENTIFICATION OF OTHER TARGETS OF GAIT PATHWAY

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DEDICATION

To my parents Mr. Sree Rama Chandra Murthy Vadlamani and Mrs. Rajeshwari Vadlamani, who have supported me, taught me, and loved me, my husband Naga Dakshina Murthy Nookala and my sister Venkata Lakshmi Mantha, my friends Srikanth Mantha, Dheeraj Reddy Bheemidi, Sree Swarna Potluri, Swetha Tadepally and Phanindra Kumar Puppala for their constant support and encouragement.

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STUDY OF 3′-UTR OF INDUCIBLE NITRIC OXIDE SYNTHASE AND ID. OF OTHER TARGETS OF GAIT PATHWAY

ABSTRACT

VADLAMANI SIRISHA

Macrophages and other leukocytes play a vital role during inflammation. IFN-γ which is secreted by Th1 cells activates monocyte-macrophages and this activation leads to induction as well as suppression of certain genes expressed during inflammation. This suppression of genes may be potentially vital for resolution of inflammation. Upon IFN-γ stimulation, a Gamma-activated inhibitor of translation complex (GAIT) is formed, which binds to 3′-UTR of target mRNAs thereby silencing their translation. VEGF-A and ceruloplasmin are two proteins whose translation is suppressed by this pathway. The GAIT complex proteins have been identified as ribosomal protein L13a, glutamyl-prolyl tRNA synthatase (EPRS), NS-1 associated protein 1 (NSAP-1), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (Mazumder *et al.,* 2003; Sampath *et al.,* 2004). The cellular abundance of the proteins involved in GAIT complex formation is in excess of Cp or VEGF mRNA, so we hypothesize that there exists additional mRNAs that are subject to GAIT-mediated translational control. Hence further studies were done to investigate other possible targets of this pathway. A probable list of targets containing putative GAIT-like elements in 3′-UTR were obtained using pattern search, a bioinformatic approach (Ray and Fox, 2007). Among these targets, inducible nitric oxide synthase (iNOS) was found to be of particular interest as nitric oxide which is produced by enzymatic activity of nitric oxide synthases, is central for the function of macrophages

and also for early immune responses to invading microorganisms. Apart from iNOS, translational silencing of putative GAIT element bearing mRNAs of ADAM10, GLUT10, LITAF, GABA B receptor and mouse iNOS in their 3′-UTR was also investigated in IFN-γ treated monocytic cells which suggests that there might be additional targets of this pathway.

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LIST OF ABBREVIATIONS

ADAM A Disintegrin and Metalloprotease	Cp Ceruloplasmin
DAPK Death-associated protein kinase	eIF Eukaryotic initiation factor
EPRS Glutamyl-prolyl tRNA-synthatase	eNOS Endothelial nitric oxide synthase
FAD Flavin adenine dinucleotide	FMN Flavin mononucleotide
GABA Gamma aminobutyric acid	GAIT Gamma activated inhibitor of translation
GLUT Glucose transporter	HRI Heme regulated inhibitor of translation
IFN-γ Interferon gamma	iNOS Inducible nitric oxide synthase
LITAF Lipopolysaccharide-induced TNF factor	IP Immunoprecipitation
JAK Janus kinase	mRNA Messenger ribonucleic acid
NADPH Nicotinamide adenine dinucleotide phosphate	nNOS Neuronal nitric oxide synthase
NO Nitric Oxide	STAT Signal transducer and activator
UTR Untranslated region	VEGF Vascular endothelial growth factor
ZIPK Zipper-interacting protein kinase	

CHAPTER I

INTRODUCTION

1.1 Introduction to Translation in Eukaryotes:

Translation of mRNA into protein represents the final step in gene expression i.e. formation of a proteome from genomic information. The structure and metabolism of eukaryotic mRNAs differ substantially from that of prokaryotes.

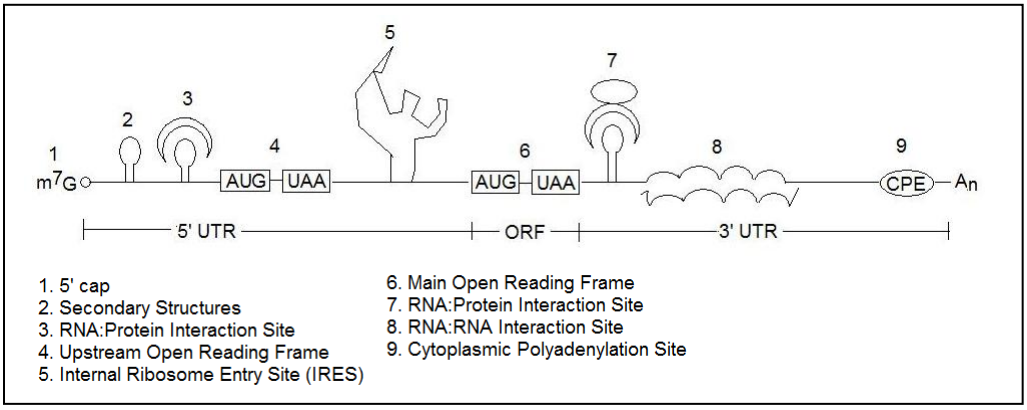

Figure 1: Nuts and Bolts of mRNA

Eukaryotic mRNAs are usually capped, polyadenylated and generally encode a single protein, i.e., they are monocistronic. The transcription and processing events are separated from translation by a nuclear membrane. Thus mature completely formed

mRNA are presented in the cytoplasm. These mRNAs possess secondary structures in both the 5′ and 3′-UTR that regulate the translation of the protein as shown in Figure 1.

Translation is accomplished by ribosomes, large ribonucleoprotein (RNP) assemblies of approximately 4mDa in association with large number of accessory factors (Zhang *et al.*, 2006). In eukaryotes mRNAs exists as mRNP (ribonucleoprotein particles). Thus mRNAs are either present as non-active mRNPs or actively translating mRNPs. Bacterial mRNAs have a rapid turnover hence it is difficult to isolate individual mRNA and translate it *in vitro*; in contrast eukaryotic mRNAs can be isolated either individually or in group and can be translated *in vitro*.

Initiation in eukaryotes is accomplished by specialized cap-binding initiation factor that binds to mRNA and positions it on the small ribosome subunit. Usually AUG after the cap is used as the initiation codon.

Translation is a process by which the information contained in the nucleotides is converted into polypeptide. This process is divided into three phases: initiation, elongation and termination. All of the three steps depend on the protein-protein, protein-RNA and RNA-RNA interactions. The first step in translation initiation is the formation of a ternary complex consisting of eIF2, GTP and Met-$tRNA_i^{Met}$. This binding is stabilized by eIF1A and eIF3. Met-$tRNA_i^{Met}$ is delivered directly to the A site unlike the other amino acids which enter P site.The mRNA processing events such as capping, splicing and polyadenylation start when the RNA polymerase II is still engaged in elongation. Various enzymes involved in RNA processing (Such as capping enzyme,

components of splicing machinery and cleavage and polyadenylation factor) are recruited by C-terminal domain of RNA Polymerase II.

1.2 Translation Control of Gene Expression: Global or Transcript Specific:

Translational control of gene expression is specially important during development and cell differentiation or during stress. It can be global translational control like phosphorylation and inactivation of eIF2α by kinases such as Heme regulated inhibitor of translation (HRI) activated by heme or iron deficiency, double-stranded RNA regulated protein kinase (PKR) activated by viral infection or double-stranded RNA, PKR-like endoplasmic reticulum kinase (PERK) activated by unfolded proteins in ER, or GCN2 activated by amino acid deficiency (GCN2) respectively (Dever, 2002; Zhang *et al.,* 2006). In case of transcript specific translation control the *trans*-acting factors usually recognize certain *cis*-acting sequences present in 3′ or the 5′-UTR of the mRNA. miRNAs also can aid in regulation of translation by hybridizing to 3′-UTR of mRNAs thereby inhibiting the elongation / termination of peptide synthesis (Olsen P H *et al.*,2002). The *cis*-acting elements are usually stem loop structures and have specific sequence requirements for silencing activity. The presence of such sequences in multiple mRNAs recognized by same RNA-binding complex suggests that this can be operated as a post-transcrptional regulon controlling the translation of certain protein families or functionally related proteins.

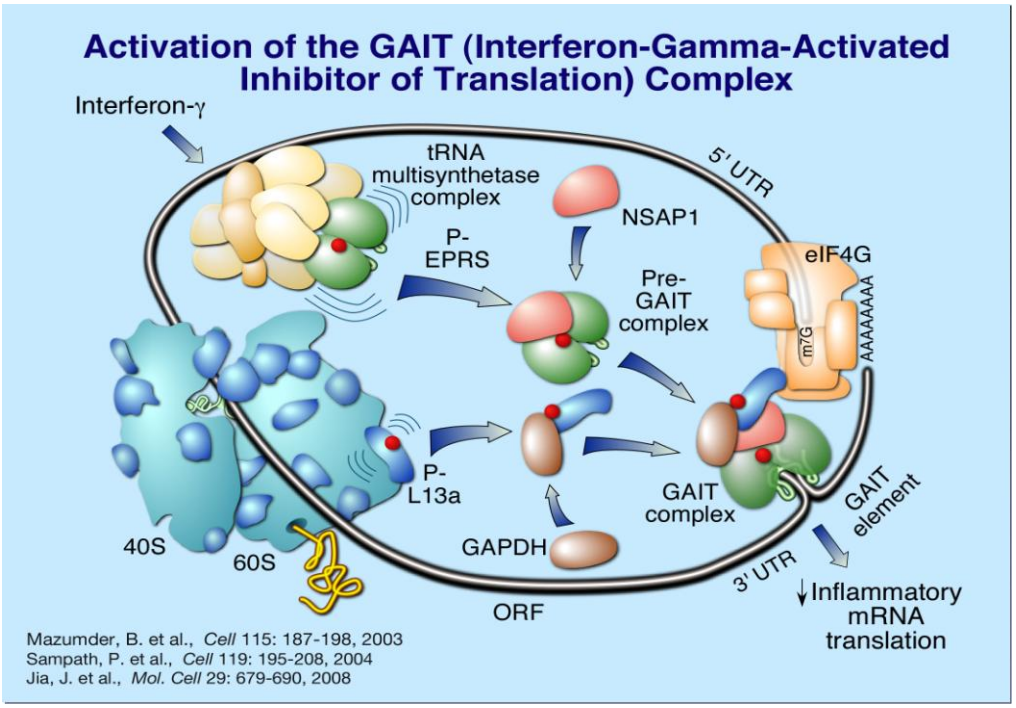

Figure 2: Activation of GAIT complex by IFN-γ treatment

Translational control of ceruloplasmin provides an example of transcript-specific translational silencing. Ceruloplasmin mRNA is circularized by its binding of poly(A) binding protein (PABP) to the poly(A) tail in the 3′-UTR and to eIF4G during the translation initiation process. Upon stimulation by IFN-γ a heterotetrameric GAIT complex is formed which binds to the GAIT element present in 3′-UTR of Cp and phosphorylated L13a which is a part of heterotetrameric complex binds to eIF3 binding site of eIF4G thus blocking the assembly of 48S ribosomal complex (Sampath P *et al.,* 2004, Kapasi P *et al.,* 2007). Similarly a gene which plays an important role in angiogenesis i.e., VEGF-A was also found to be translationally controlled by GAIT pathway (Ray and Fox 2007).

CHAPTER II

GAIT PATHWAY

2.1 GAIT Complex Components and Structure of GAIT element:

The GAIT complex components have been identified as glutamyl-prolyl tRNA-synthatase (EPRS), ribosomal protein L13a, NS-1 associated protein 1 (NSAP1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mazumder *et al.*, 2003; Sampath *et al.*, 2004). EPRS is phosphorylated and released from multisynthetase complex and phosphorylated L13a is released from the large 60S ribosomal subunit.

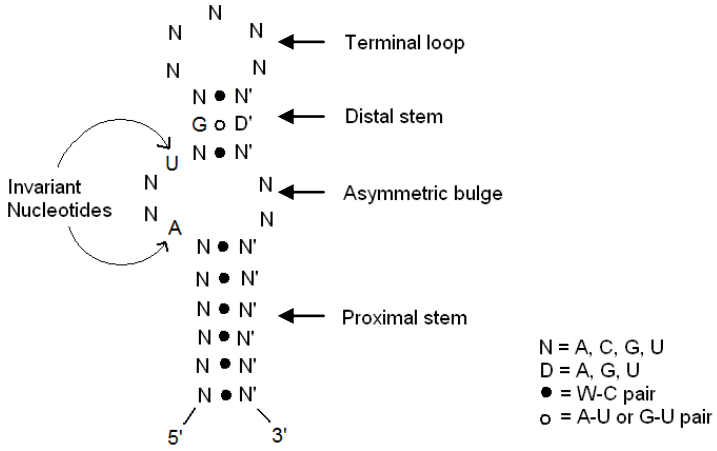

Figure 3: Predicted Structure of GAIT element (Sampath *et al*, 2003)

GAIT complex is assembled in two stages: in the first step after 2 to 4 hrs of IFN-γ treatment EPRS binds NSAP1 to form pre-GAIT complex which cannot bind the GAIT element. In the second step phosphorylated L13a and GAPDH join the pre-GAIT complex after 12 hrs to form active GAIT complex (Jia *et al.,* 2008) (Figure 2).

Sampath et al. have shown that a 29 nucleotide (nt) element found in 3′-UTR of Cp mRNA is the minimal sequence required for binding of the GAIT-complex and subsequent silencing. Mutation analysis has shown that GAIT element has certain structural as well as sequence requirements. The element has a 5-nt terminal loop separated from an asymmetric bulge by a short 3-nt stem. The entire structure is held by a proximal 6-nt double helical stem. It has invariant U and A nucleotides in the asymmetric bulge (Sampath *et al.,* 2003, Mazumder *et al.,* 2003).

2.2 Ceruloplasmin and VEGF-A regulation by GAIT pathway:

Ceruloplasmin is a glycoprotein which is primarily synthesized by liver in adult humans. IFN-γ stimulation of ceruloplasmin is cell type specific and it contributes to the defense responses by its ferroxidase activity. IFN-γ stimulates ceruloplasmin production differently in several cell types (Mazumder *et al.,* 1997).

IFN-γ induces ceruloplasmin synthesis in U937 monocytic cells (Mazumder and Fox, 1999) and its protein expression is halted in a delayed silencing process. This silencing process, apart from binding of GAIT complex to mRNA, requires interaction between eukaryotic initiation factor 4G, poly(A) binding protein and poly(A) tail (Mazumder *et al.,* 2003).

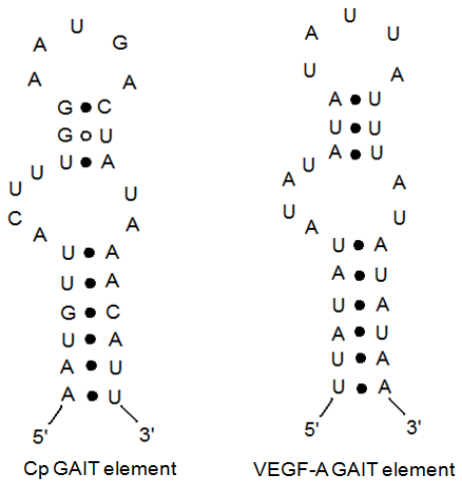

Figure 4: GAIT element structure of Ceruloplasmin and VEGF

The very high cellular abundance of four GAIT complex components and the stoichiometric release of L13a and EPRS from respective parent complexes is much in excess of Cp mRNA. Hence it has been speculated that there exists additional targets which are regulated by GAIT pathway. Further investigations were done to identify potential targets of GAIT mediated pathway. The 3′-UTR database was searched using the Cp GAIT element sequence and secondary structure information and VEGF-A was found to be of particular interest (Ray and Fox, 2007).

VEGF-A expression is regulated by hypoxia and cytokines at the levels of transcription, mRNA stability and translation. GAIT mediated pathway of VEGF-A regulation is the first identified negative regulatory mechanism. It has been shown in our laboratory that GAIT complex suppresses VEGF-A expression in IFN-γ activated monocytic cells such as U937 and PBMCs by inhibiting its translation (Ray and Fox, 2007).

2.3 Mfold and Patsearch programs to predict the structures of putative GAIT targets:

The mfold program for RNA folding was used to predict the secondary structures of RNA when folded under simulated physiological conditions. This program folds the RNA at 37°C and it calculates the minimum free energy for any particular folding and the base pairs that it must contain. This program also gives the flexibility to use certain constraints to fold the RNA with a limitation that the free energy of the newly formed structure does not differ too much from the one formed under unrestricted conditions.

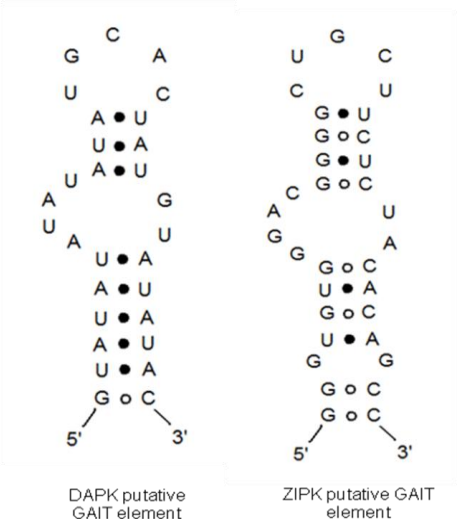

Figure 5: mfold predicted structures of DAPK and ZIPK GAIT elements

Figure 5 shows the putative GAIT elements present in DAPK and ZIPK which have been experimentally verified (Mukhopadhyay R. et al., *Molecular Cell* In press) as GAIT targets. In ZIPK GAIT element it has been observed that it has four base pairs in the distal stem unlike the classical Cp GAIT element and it also had two G-C base pairs in this distal stem which was thought to be destabilising the Cp GAIT as found in the mutational analysis. It is held by a clamp of two G-C base pairs and has an G-G bulge in the proximal stem.

A GAIT element pattern, based on the sequence requirements and secondary structure of Cp GAIT element, was used to query a non-redundant 3′-UTR database containing 217 135 sequences using PatSearch. Patsearch is a folding energy independent, pattern matching algorithm that searches for nucleotide sequences as well as secondary structures, that is stems and loops of defined length (Ray and Fox 2007). When the 5′-UTR was used to query for presence of GAIT like elements it gave only 5 results out of 190 432 sequences, showing that this element was present more abundantly in 3′-UTR with specific roles. VEGF-A GAIT element was also found through this search and it was also particular interest as no negative regulation of VEGF-A was known in literature.

Table 1: Partial List of Pattern Search for GAIT element in Human 3′-UTRs

Query:
r1={au,ua,gc,cg}
r2={au,ua,gu,ug}
p1=6...6 A 2...2 U p2=1...1 p3=1...1 p4=1...1 5...5 r1~p4 r2~p3 r1~p2 2...2 r1~p1

PATSEARCH RESULTS

3HSA012637 : [3706,3734] :	tttatgatttgagttggcctcttcataaa	KIAA0448 (heparan sulfate 2-O-sulfotransferase 1)
3HSA018230 : [257,285] :	ccagggacctctctccgggggctccctgg	NDRF NeuroD-related factor
3HSA029633 : [1228,1256] :	atatatatatatagtgtttgtatatatat	KIAA1849 (hypothetical protein FLJ10324)
3HSA071070 : [2118,2146] :	tttataaacttactgactgtaaatataaa	HBE120
3HSA059964 : [6826,6854] :	atattaaatttatattaaataggtaatat	KIAA2014 (formin-like 3 (FMNL3), transcript variant
3HSA011233 : [697,725] :	tttaaaatgtgtatagtatgcattttaaa	ZnF20
3HSA015153 : [1083,1111] :	ccagtgagctctccccttgggatcactgg	GABA-B receptor
3HSA057132 : [1010,1038] :	tggtaaattttatgtagtatattttacca	HSC3 (DnaJ (Hsp40) homolog, subfamily B)
3HSA021418 : [2684,2712] :	atatatatatatatataatatgtatatat	nuclear factor RIP140
3HSA021420 : [632,660] :	tttattatttttgctcatcaaaaaataaa	mitogenic oxidase MOX1
3HSA040055 : [1569,1597] :	tataaaaaattaaatgatttaaatttata	GLUT10
3HSA040874 : [1122,1150] :	atatatatatatatgatatatatatatat	ARG147 (DEAD-box containing helicase protein)
3HSA057588 : [374,402] :	atgtaaatgtattggcacagtgcttacat	BAALC (brain and acute leukemia, cytoplasmic)
3HSA031254 : [706,734] :	atatatatatatatgatatatatatatat	FLJ22060 fis (DEAH (Asp-Glu-Ala-His) box
3HSA055996 : [537,565] :	gtgcttaaatattctaggagtagaagcac	highly similar to Mus musculus mRNA for gasdermin.
3HSA071874 : [9,37] :	ggtactactttgaatctgttactagtacc	similar to Rattus norvegicus dynamin-like protein
3HSA077382 : [6,34] :	tcattgagttcggtgcatctggccaatga	Niemann-Pick disease, type C2, precursor
3HSA082033 : [238,266] :	ttattaaagtattaatatagttttaataa	selenoprotein K
3HSA047090 : [395,423] :	acaaaaagattttattaaagatattttgt	NG22 protein
3HSA088147 : [984,1012] :	taagtgacatttaatgagtgaagcactta	guanine nucleotide exchange factor for Rap1
3HSA091211 : [358,386] :	**ttatatatatatatattatatatatataa**	**protein for MGC:70609 (Homo sapiens VEGF)**
3HSA000816 : [933,961] :	atatatatatatatataatgtgtatatat	heparan sulfate-N-deacetylase/N-sulfotransferase mRNA
3HSA070141 : [639,667] :	attgtgaaatattaaaataattccacaat	solute carrier family 2 (facilitated glucose transporter)
3HSA066209 : [2065,2093] :	aaatatagttaaatcctctttaaatattt	triadin
3HSA003031 : [78,106] :	**aatgttactttggaatgactataaacatt**	**ceruloplasmin**
3HSA003724 : [1141,1169] :	gtatatatatatatgcactatgtatatac	DAP-kinase
3HSA038709 : [1735,1763] :	attgaaaagtataaaatttatctttcaat	DKFZp547M114 (contactin 3 (plasmacytoma associated))
3HSA070530 : [5589,5617] :	atatataatttaaataatttaaaatatat	spectrin repeat containing, nuclear envelope 1

Ray *et al.* have performed a bioinformatic/candidate gene approach to find out the possible targets of GAIT-mediated pathway. In this approach U937 cells were treated with IFN-γ for indicated time periods and the cell lysate was immunoprecipitated with α-EPRS. Then total RNA was isolated from it and was subjected to reverse transcription using oligo(dT) primers, this cDNA was sent for Microarray analysis. Among the results obtained inducible nitric oxide synthase was found to have highest mean fold enrichment IP/Total RNA, hence it was further investigated to test for presence of GAIT element in its 3′-UTR.

Further insight into the GAIT mediated pathway was obtained by Mukhopadhyay *et al* with the finding of death-associated protein kinase that activates zipper-interacting protein kinase which further phosphorylates L13a on Ser[77.] This phosphorylated L13a is released from the ribosome thereby forming the GAIT complex and silencing the translation of target proteins. Thus the mRNAs of the kinases of GAIT mediated pathway are themselves translationally silenced by this pathway hence it constitutes a autoregulatory mechanism (Mukhopadhyay R *et al., Molecular cell* in press).

CHAPTER III

INDUCIBLE NITRIC OXIDE SYNTHASE

3.1 Structure and Function of Inducible Nitric Oxide Synthase:

Nitric oxide synthases (NOS) are present as dimers in eukayotes. They are of three types (a) inducible NOS (b) neuronal NOS and (c) endothelial NOS encoded by three different genes. iNOS has been implicated in the pathogenesis of various diseases such as Alzheimer's disease, tuberculosis, asthma, glaucoma, inflammatory bowel disease, arthritis, stroke and septic shock (Jagannath C *et al.*, 1998).

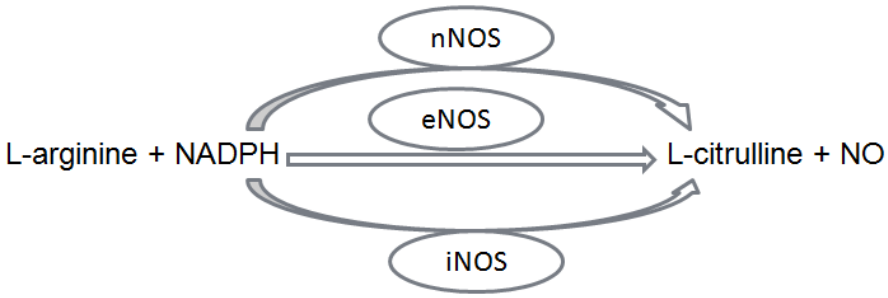

Figure 6: Enzymatic activity of Nitric oxide synthases

Macrophages are important for early immune responses against invading microorganisms and the production of nitric oxide (NO) is central to this function. NO is generated by

inducible nitric oxide synthase (iNOS or macNOS or Type II NOS) following exposure to certain cytokines, such as interferon-γ (IFN-γ) as depicted in Figure 7. IFN-γ a Th1 cytokines activates cytotoxic T lymphocytes, natural killer (NK) cells and macrophages. The IFN-γ receptor signals through the Janus kinase (JAK) family and signal transducers and activators of transcription (STAT) proteins. Receptor occupation and dimerization induces the phosphorylation of associated STATs. Activated STATs dimerize and translocate to the nucleus where they increase expression of the transcription factor, IRF-1, that, in turn binds to specific DNA elements in the iNOS gene promoter region to increase iNOS gene expression (Massa P. T *et al.*, 1998; Fehr T., *et al.*, 1997).

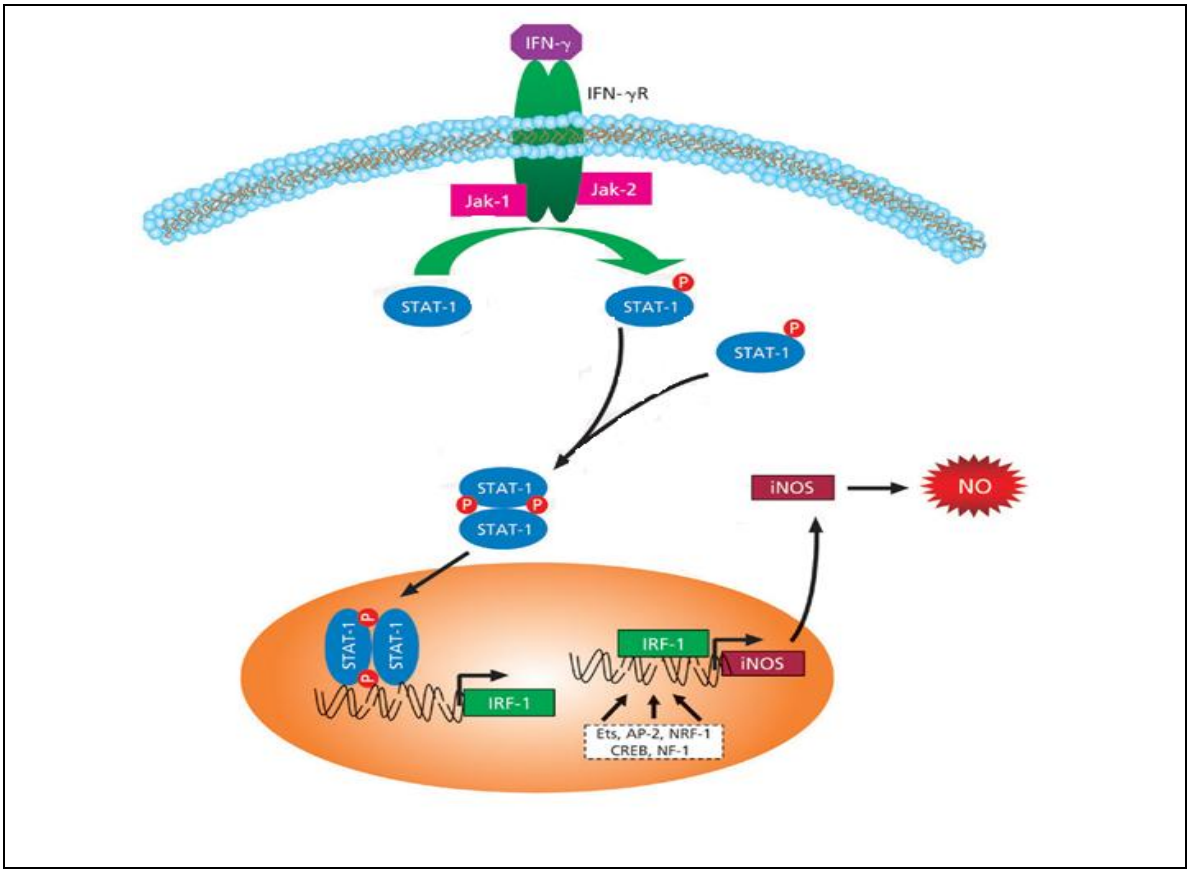

Figure 7: Transcritional upregulation of iNOS by cytokines

(Massa P. T *et al.*, 1998; Fehr T. *et al.*, 1997)

Inducible nitric oxide synthases catalyse the conversion of L-arginine to L-citrulline which has different cofactor requirements. This is a two step reaction involving a two electron oxidation in first step to form N-hydroxy-L-arginine and a second step of three electron oxidation to form L-citrulline and the free radical nitric oxide (Figure 8).

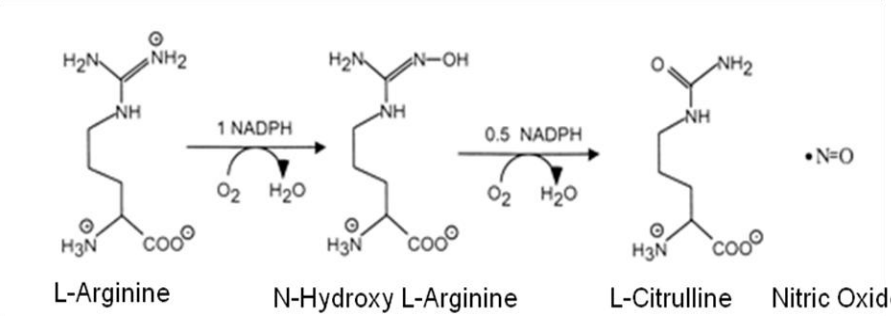

Figure 8: Chemistry of iNOS enzymatic activity

iNOS is a soluble enzyme that, unlike eNOS and nNOS, does not require elevated intracellular Ca^{2+} levels for activation. The NOS enzymes have three different domains: a reductase domain, a calmodulin-bindng domain and an oxygenase domain (Figure 9).

a) The reductase domain: This domain consists of FAD, FMN moieties and transfers electrons from NADPH to oxygenase domain. Here one reductase domain will aid in transfer of electrons to oxygenase domain of another subunit.

b) Calmodulin binding: This binding is required for the activity of the enzyme and it detects changes in intracellular calcium levels.

c) The oxygenase domain: The binding domains for heme, arginase and tetrahydrobiopterin are present in this domain. It also catalyses the conversion of arginine into citrulline and NO.

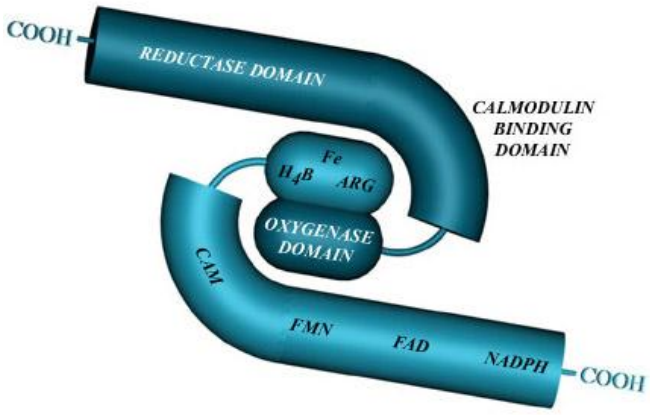

Figure 9: Structure of iNOS dimer (Adapted from www.sgul.ac.uk/dept/immunology)

The human iNOS contains 26 exons and encodes a 131 KDa protein. Intracellular iNOS largely exists as a soluble cytosolic fraction. It is expressed in macrophages, monocytes, leukocytes, endothelium, smooth muscle, neutrophils, retinal pigmented epithelium, astrocytes, microglial, hepatocytes, kupfer cells, fibroblasts and mesangium (Berthlot S *et al.,* 1999). It has been known that iNOS is transcriptionally upregulated by LPS and certain cytokines in murine cell line. There are no reports on the translation regulation of inducible nitric oxide synthase in monocytic cells, hence we further investigated the effect of IFN-γ treatment and its relationship to GAIT-mediated pathway in human monocytic cell line i.e. U937 cells.

From the results of bioinformatic approach which has given a probable list of mRNA with putative GAIT like elements, inducible nitric oxide synthase was found to be the gene with highest mean fold enrichment IP/Total RNA (Ray and Fox 2007). Thus the detailed sequence analysis of iNOS mRNA was done which shows that it has 'CA-rich' nucleotide sequence ahead of mfold predicted GAIT like structure. This suggests that like VEGF-A, iNOS may also be regulated by both IFN-γ and hypoxia. We further

investigated the effect of iNOS 3′-UTR in translational control with a primary goal to study the effect of IFN-γ activation in monocytes.

3.2 Study of 3′-UTR of human and Mouse iNOS for presence of GAIT element:

It has been reported that VEGF-A 3′-UTR has a sequence which behaves as a riboswitch that integrates signals from hypoxia and IFN-γ to regulate VEGF-A translation in myeloid cells (Ray P. S. *et al.,* Nature In press). The VEGF-A 3′-UTR has a hypoxia stability region (HSR) that binds hnRNP L and confers hypoxia mediated stability to mRNA. hnRNP L protein has high affinity to bind to CA-repeat sequence (Hui J *et al.,* 2003), VEGF-A mRNA has a A/C rich sequence which corresponds to hnRNP L binding site.

Previous studies on mouse iNOS 3′-UTR has shown that inflammation effects the interaction of heterogenous ribonucleoproteins i.e. hnRNP L and hnRNP I/PTB with mouse iNOS 3′-UTR (Soderburg M. *et al.,* 2002). The 3′-UTR sequences of Mouse and human iNOS are 473 and 477 bp respectively. The Mouse iNOS hnRNP L binding site is 434-456bp and a mfold predicted GAIT element of mouse iNOS was found to be located from 435-467bp (Figure 10). The hnRNP L binding region was encompassed within the binding site of GAIT element. A further alignment of mouse iNOS 3′-UTR region with that of human iNOS 3′-UTR has shown that there is about 83% of sequence homology between them particularly in the extreme 3′ region where the mouse hnRNP L binding domain was located.

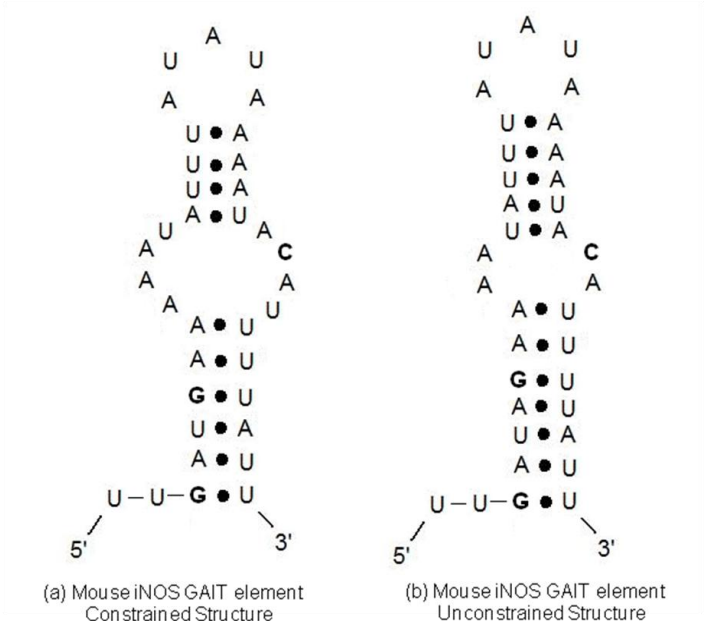

Figure 10: mfold predicted putative Mouse iNOS GAIT-like element

Thus a further investigation was done on human iNOS 3′-UTR was done to see if it able to confer translational silencing to Luciferase reporter construct.

Mouse iNOS 3′-UTR sequences of GAIT-like element, hnRNP L binding sequence

Mouse iNOS 3′-UTR (433-467):
433 TGATGAAAAATATTTATATAAAATACATTTTATT 467

Mouse hnRNP L binding site:
433 TGATGAAAAATATTTATATAAAA 455

Mouse GAIT like structure:
434 GATGAAAAATATTTATATAAAATACATTTTATT 467

Sequence homology between Mouse iNOS and Human iNOS 3′-UTR

Query (mouse iNOS)
Subject (Human iNOS)

```
Query 409CTTGGGCCTTTCCTGTATAATTCCTTGATGAAAAATATTTATATAAAATACATTTTATTTTAATC473
      |||||| ||| ||||||| ||||||||||  | ||||||| || || | ||||||| |||||||
Sbjct 414CTTGGGTCTTCCCTGTATGATTCCTTGATG-GAGATATTTACATGAATTGCATTTTACTTTAATC477
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3.3 Study of other targets for presence of GAIT-like element:

Earlier data on GAIT mediated silencing has shown that the protein expression is silenced in U937 cells treated with IFN-γ for 24hr (Mazumder *et al.,* 2003; Ray and Fox, 2007). Based on the results obtained from pattern search analysis and Bioinformatic approach a list of proteins associated with inflammation and which had a high IP/Total RNA in RIP-ChIP analysis were identified for further investigation.

A preliminary western blotting was done to observe the protein expression pattern in IFN-γ treated U937 cells at 0,8 and 24hr for ADAM10, GLUT10, LITAF, GABA B receptor and mouse iNOS.

γ-aminobutyric acid is a major neuroinhibitory transmitter in the mammalian central nervous system but recent studies have shown that it is located in nonneuronal cells in peripheral tissue(Osawa Y., et al., 2006). It is composed of two subunits and is functional only when both the subunits are expressed and the heterodimer assembly is complete. In the present study we investigated for the presence of $GABA_B$ receptor expression in monocytic cells. Further studies may provide insight into functional characerization for its presence in monocytic cells.

CHAPTER IV

METHODS

Reagents

Rabbit reticulocyte lysate, methionine-minus amino acid mixture and RnaseIn were purchased from Promega (Madison, WI, USA). Human IFN-γ from R&D systems (Minneapolis, MN, USA), [35S] methionine was purchased from Perkin-Elmer for in vitro translation (translation grade).

Cell Culture

Human U937 monocytic cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin and 2mM L-glutamate. Cells were maintained in a humified atmosphere containing 5% CO_2 at 37º C. U937 cells were treated with 500U/ml of IFN-γ for the times indicated. The iNOS 3´UTR was amplified from total RNA obtained from U937 cells and cloned into pGEMT vector (Promega, Madison, WI, USA). The DNA fragment was inserted downstream of FLuc, in the vector pSP64-FLuc to obtain pSP64-FLuc-iNOS 3´UTR.

Preparation of cell extracts

Two days before treatment, the cells were passaged in a dilution of 1:5 per flask. Cells were seeded on 60-mm Petridish, they were preincubated in 0.5% serum for 1 hour and treated with 500U/ml of IFN-γ for an additional 8 and 24 hours, harvested by scraping and the cells were washed in ice-cold D-PBS. The cells were collected by centrifugation at low speed and the cell pellet was suspended in a mixture of 50mM Tris (pH 7.6), 50mM NaCl, 1mM PMSF and 1mM DTT and was passed through 26-gauge syringe several times and incubated on ice for 10 minutes, ultracentrifuged at 100,000 X g for 30 minutes. The protein concentration was assayed using Biorad reagent with bovine serum albumin as standard, adjusted to 1mg/ml and 500ng was used in the in vitro translation reaction.

Total RNA isolation and Semi-Quantitative RT PCR

Total RNA was isolated from cells treated with IFN-γ using Trizol reagent according to the manufacturer's instructions. Total RNA was first extracted using Trizol followed by a chloroform extraction. RNA was then precipitated from the aqueous phase by mixing with isopropanol, the precipitate was washed with 75% ethanol and then suspended in RNase free water. To eliminate any possibility of DNA contaminations in the RNA preparations, samples were digested with 1U DNase I and 1μl of DNase buffer 10X (Promega, Madison, USA) per μg of RNA was incubated with each sample for 15 minutes at room temperature. The reaction was stopped by adding stop reaction mix. Phenol:Chloroform extraction was performed to separate RNA followed by precipitation with sodium acetate and ethanol 100%. The precipitate was washed with 75% ethanol

and suspended in RNase free water. Then 1μg of RNA was reverse transcribed using Oligo(dT) primers. cDNA was subjected to PCR amplification by primers designed for amplification of full length 3′-UTR. The optimal number of cycles and the temperature for amplication, annealing time, extension time were determined in a first few preliminary experiments designed to detect iNOS expression in IFN-γ treated U937 cells. It was then normalized to amplified β-actin cDNA.

Construction of vectors containing iNOS 3′UTR and Fluc in pSP64

The iNOS 3′UTR consisting of 475 bp was cloned with BamHI and SacI restriction sites at the 5′ and 3′ ends respectively . The 495bp was then inserted into pSP64 in the corresponding restriction site. The Luciferase gene was cut from pcDNA3 which has HindIII and XhoI restriction ends at 5′ and 3′ ends respectively and cloned into pSP64 vector containing iNOS 3′UTR between HindIII and SalI sites. Thus the recombinant reporter gene containing the iNOS 3′UTR downstream of FLuc was made. A set of intron spanning primers were designed to ensure that there is genomic DNA contamination in the total RNA isolated.

The following are the set of primers used for amplification of iNOS cDNA isolated from Total RNA of IFN-γ treated U937 cells:

FP with Stu I (5′-3′): TAA TAGGCCTCT ACA GGA GGG GTT AAA

FP with BamHI (5′-3′): TTAAGGATCCCCTACAGGAGGGGTTAA

RP with Sac I (5′-3′): GGG CGAGCTCGT GAT TAA AGT AAA ATG

iNOS intron spanning FP (5′-3′): GCA TGA CCT TGG TGT TTG GGT

iNOS intron spanning RP (5′-3′): TTC AAT TTC AGC TTG GCA GCC

In vitro transcription of iNOS 3′-UTR

pSP64 plasmid constructs containing VEGF-A 3′-UTR or iNOS 3′-UTR sequences downstream of FLuc were linearised by digesting it with BamH1 and transcribed in vitro using the mMessage mMachine transcription system (Ambion, Austin, TX, USA) to generate capped poly(A) tailed RNAs. Capped RLuc was similarly transcribed from the plasmid pRL-SV40 (Promega).

In vitro translation of mRNA by reticulocyte lysate

For in vitro translation (12.5μl reaction) 200ng of RLuc, 300ng of FLuc-iNOS 3′-UTR, 40units of RnaseIn, 8.75μl of rabbit reticulocyte, 0.3μl of minus Met amino acid mixture, 0.75μl of 35S Met were incubated for 90 minutes at 30º C. Capped poly(A) template RNAs were translated in RRL (Promega) in the presence of a methionine-free amino-acid mixture and translation-grade [35S] methionine (Perkin Elmer, Boston, MA, USA). Cyotsolic extract (500ng protein) from untreated of IFN-γ treated cells were added to translation reactions. A 5μl reaction was resolved by SDS-PAGE (10% polyacrylamide), fixed in 40% methanol and 10% acetic acid solution for 15- 20 minutes and visualized by phosphorimaging.

Immunoblot analysis of iNOS

Cell lysates were denatured under nonreducing conditions, and resolved on SDS-PAGE. After transfer the blot was probed with anti-human iNOS antibody and HRP conjugated anti-rabbit secondary antibody, and detected by ECL (Amersham, Arlington Heights, IL, USA). Immunoblotting was also done by anti-VEGF as a positive control and anti-GAPDH to ensure equal loading.

Nitrite Assay

Nitric oxide is a molecular mediator of many important physiological processes such as vasodilation, inflammation, thrombosis, immunity and neurotransmission. Owing to its very short half-life (milliseconds) accumulation of stable degradation products such as Nitrite or Nitrate are taken as an index to measure the NO levels. Griess reagent is used in one of these methods to assay for nitrite (NO_2^-). This assay is based on diazotization reaction as described by Greiss. In this reaction sulfanilamide is converted into a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl)ethylenediamine, forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 548nm. This can be used to detect NO_2 in a variety of biological systems such as plasma, urine and tissue culture medium. Nitrite standards were prepared in the range of 1-100μM using sodium nitrite. For each reaction 100μl of Greiss reagent, 300μl of the nitrite-containing sample and 2.6mL of deionized water were mixed in a cuvette. This mixture was incubated at room temperature for 30 minutes. A reference sample was made by mixing 100μl of Greiss reagent with 2.9mL of water. The absorbance of nitrite containing sample was measured

at 548nm relative to the reference sample. Different concentrations of sodium nitrite solution were prepared and whose absorbance was taken at 548 nm; A standard curve was plotted for different concentrations and the observed absorbance. The unknown concentrations of the samples were calculated from the standard curve and a graph representing the trend of NO levels in the U937 cells treated with IFN-γ and untreated cells.

CHAPTER V

RESULTS

IFN-γ stimulated production of iNOS mRNA by the human macrophage cell line U937

Previous studies in our lab have shown that Cp and VEGF mRNA levels are increased by IFN-γ treatment which contain functional GAIT element. To determine whether or not iNOS mRNA is similarly stimulated by IFN-γ, U937 cells were treated with IFN-γ for 0, 8 and 24 hours and the total RNA was isolated using Trizol reagent.

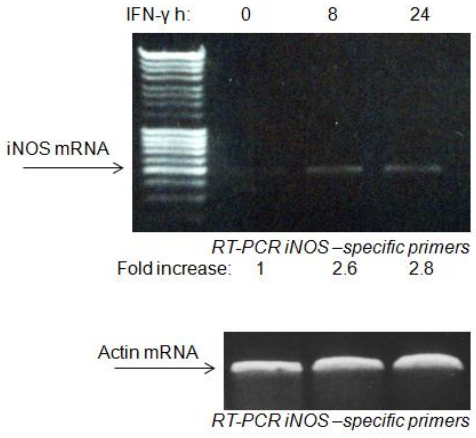

Figure 11: RT-PCR of iNOS gene and GAPDH control using gene specific primers

RT-PCR was performed using iNOS specific and Actin primers. Semi-Quantitative PCR has shown a 2.6-fold increase in iNOS mRNA at 8 hrs and 2.8-fold increase at 24hrs; Once the message levels of iNOS mRNA was determined at different time points, the next step was to check for its protein levels *in vivo*.

To determine the function of iNOS 3′UTR in translational silencing in IFN-γ treated monocytic cells

To investigate the role of iNOS 3′UTR in translational silencing endogenously, cells were treated with IFN-γ for the indicated time periods and cell lysates were processed in Laemlli gel-loading buffer in the presence of reducing agent. Lysates were then subjected to immunoblotting with anti-iNOS. It has been observed in RT-PCR that the message levels are upregulated in response to IFN-γ treatment by 2.6- and 2.8-fold respectively.

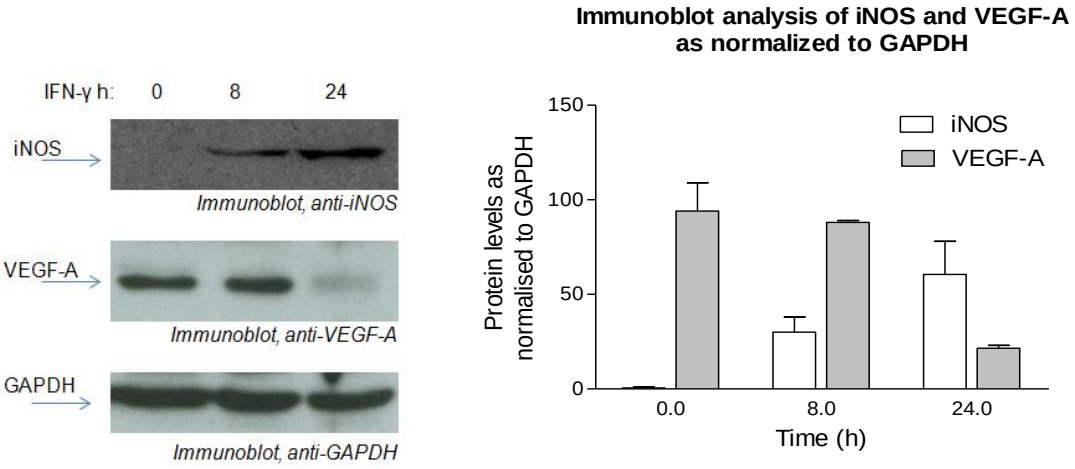

Figure 12: Western blotting result of iNOS gene using GAPDH and VEGF-A as controls

Likewise cellular iNOS protein expression was also increased at 8hr but as in case of VEGF-A or Cp there was no down-regulation seen at 24 hrs. In VEGF-A and Cp which contain functional GAIT element, the protein synthesis was silenced by more than 50%

after 24hrs. To conform the presence of functional GAIT complex in these cell lysates, VEGF-A protein levels were assessed at 0, 8 and 24 hrs and GAPDH was used as a loading control. It has been observed that VEGF-A protein evels has gone down at 24hr in contrast to iNOS expression which has been upregulated.

To check for translation silencing in rabbit reticulocyte lysate

To check for the translational levels of iNOS in cell-free rabbit reticulocyte lysate in the presence of ^{35}S-Met; iNOS 3′-UTR was cloned downstream of Firefly Luciferase open reading frame inserted in pSP64 vector and its translation levels were seen in *in vitro* translation system. A capped, poly(A)-tailed RNA transcribed in vitro and used as a template for translation in rabbit reticulocyte lysate (RRL) system. The FLuc-Cp GAIT-A_{30} was used as a positive control to confirm the formation of functional GAIT complex in cell lysate.

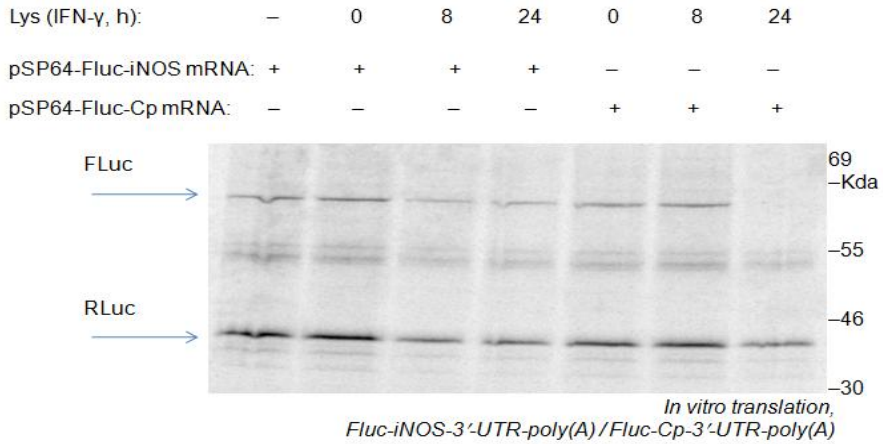

Figure 13: In vitro translation of pSP64-Fluc-NOS and pRL in rabbit reticulocyte lysate

Lysates from cells treated with 24 hrs of IFN-γ have shown to inhibit Cp synthesis but not FLuc-iNOS 3′-UTR in conformance with what was seen in western blot. Translation

of capped *Renilla* Luciferase (RLuc) RNA lacking the 3′-UTR segment was used as a control template, was not inhibited.

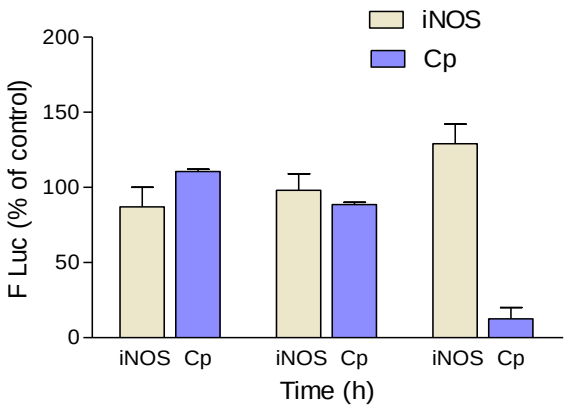

Figure 14: Fluc quantified by densitometry normalized to Rluc and expressed as a percent of control without cell lysate

Inhibition of a protein synthesis by GAIT complex is highly sequence specific as seen in case of Cp and VEGF-A. Lack of inhibition of iNOS protein suggests that it may lack the specific sequence required for silencing. Translation upregulation was seen during the time course of 0, 8 and 24hr in FLuc-iNOS 3′-UTR in contrast to translational silencing of VEGF-A or Cp as seen from the previously published work.

Assay for Nitrite in cell culture supernatant

To investigate the activity of iNOS in vivo, assay for nitrite levels in culture supernatants was performed. Nitric oxide is readily oxidized to Nitrite and this can be assayed using Greiss reagent. As described the assay was performed according to the protocol and the nitrite levels were measured using sodium nitrite as standard at 548nm. A standard curve was generated starting with 100nM sodium nitrite concentration with increasing concentrations. Readings were taken in duplicate, similarly readings for the sample

preparations was also taken in duplicates and compared with that of sodium nitrite containing serial dilutions.

Table 2: Concentrations of Sodium Nitrite standard and absorbances at 548nm

Conc. of sodium Nitrite(nM)	Abs (at 548nm)
0	0
100	0.050
250	0.132
500	0.232
700	0.338

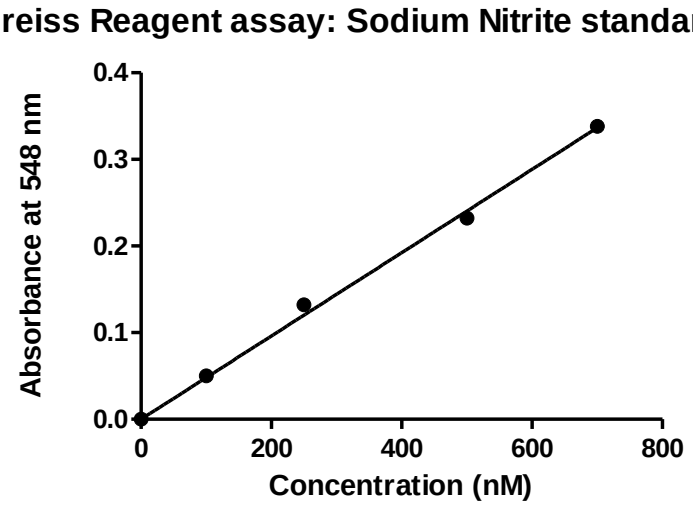

Figure 15: Standard curve of Sodium Nitrite concentration plotted against mean of the absorbance at 548nm

U937 cells were either treated with IFN- γ for 0, 8 and 24 hr respectively and the nitrite assay was performed using greiss reagent. Cell were treated in duplicates either with IFN-γ, without IFN-γ as a control set.

Table 3: Nitrite levels in cell culture supernatant in two different sets

Time (hr)	Mean of Set 1 (IFN-γ treated)	Set 2 (Untreated cells)
0	15.497	8.265
8	27.896	12.398
24	86.788	20.664

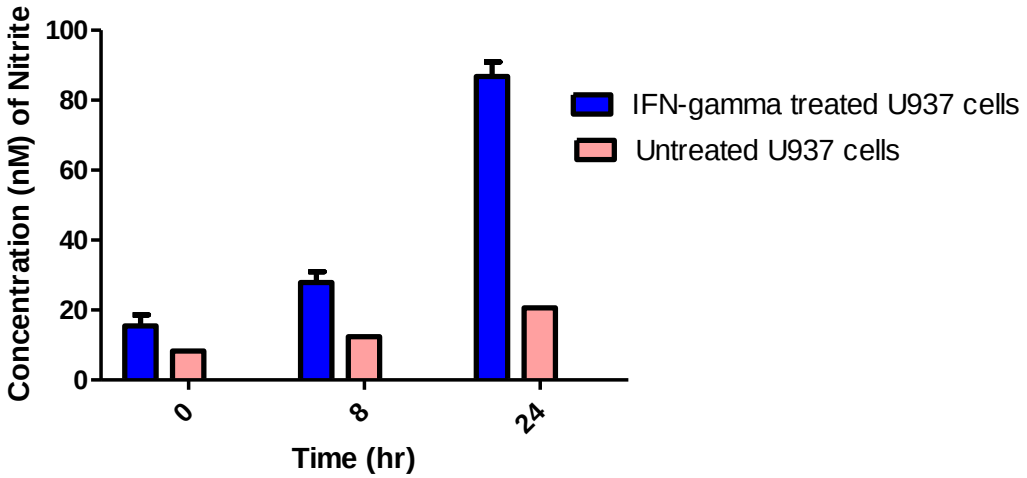

Figure 16: Statistical presentation of NO levels in U937 cells treated with IFN-γ and Untreated Cells

Western blotting of other targets containing GAIT-like elements

U937 cells were treated with IFN-γ for 0, 8 and 24 hr and the cell lysate was subjected to western blot analysis using different antibodies as mentioned. Analysis for VEGF-A protein levels using α-VEGF-A has shown that its expression is silenced at 24hr which shows that the functional GAIT complex has formed. The same cell lysate was used to blot the remaining targets ADAM10, GLUT10, LITAF, and $GABA_B$ using respective antibodies.

Similarly RAW 264.7 cells were treated IFN-γ for 0, 8 and 24 hr and the cell lysate were tested for iNOS expression. Mouse iNOS in RAW 264.7 cells and $GABA_B$ in U937 cells

were found to be down-regulated by IFN-γ at 24hr. ADAM10, GLUT10 and LITAF protein levels were upregulated with time at 0, 8 and 24hr.

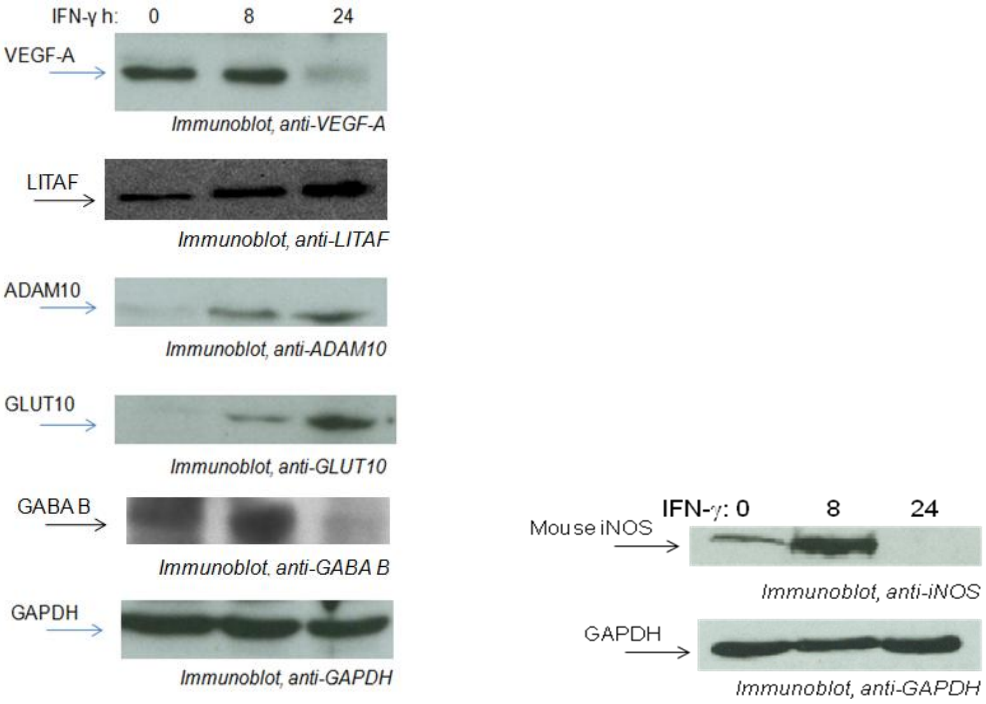

Figure 17: The protein expression of the targets containing putative GAIT-like elements as found from pattern search and Bioinformatic approach

CHAPTER VI

DISCUSSION AND FUTURE INVESTIGATIONS

Nitric oxide which is produced by the activity of NO synthase plays a vital role in defense against various pathogens in macrophage-mediated immune response. iNOS is transcriptionally upregulated in RAW cells and human monocytes by certain cytokines such as IFN-γ, TNF-α, IL-1β and LPS. Previous studies done on Mouse iNOS 3′-UTR has shown the presence of hnRNP L binding to it (Malin *et al.*, 2002) and regulation of its expression during inflammation. Michael Zuker mfold program has shown that that mouse iNOS 3′-UTR has GAIT-like structure and this is encompassed within the hnRNP L binding region. Human iNOS which is about 83% similar to mouse iNOS has a similar CA- repeat sequence and was also found to be transcriptionally upregulated by IFN-γ as seen from the message levels of iNOS in IFN- γ treated U937 cells. But the protein levels were also consistently increasing in-line with the message levels.

The GAIT element present in the 3′-UTR of ceruloplasmin and VEGF-A mRNA mediates in translational silencing of its protein expression (Mazumder *et al*, 1999; Ray and Fox 2007). The proteins involved in GAIT complex formation were found to be much in excess of Cp and it has been speculated that presence such elements in multiple

mRNAs would allow it to be operated as an post-transcriptional regulon controlling the expression of certain protein families. Functional GAIT elements have also been identified in DAPK and ZIPK which are two kinases involved in this pathway (Mukhopadhyay R. et al., *Molecular Cell In press*) creating a autoregulatory mechanism. Using Cp GAIT element to query the 3′-UTR database other targets containing GAIT-like elements were listed and among these some of the targets associated with inflammation were further investigated to confirm for the presence of functional GAIT element in them. Western blotting data has shown that $GABA_B$ receptor and mouse iNOS may be two positive targets whose protein expression was found to be silenced at 24hrs in IFN- γ treated U937 cells.

Thus this pathway provides one of the important findings in inflammation and the clinical significance of the genes regulated by this mechanism has made this much studied subject. Uncontrolled activation and expression of certain proteins might pose risk to the cells and this pathway provides a mechanism by which this expression is controlled as seen in case of Cp and VEGF-A. Mouse iNOS and $GABA_B$ whose expression was found to be down-regulated as seen from the results of preliminary western blotting are yet to be studied further. This investigation may provide an understanding of pathological conditions associated with $GABA_B$ / Mouse iNOS and thus lead to discovery of some novel targets in this pathway.

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