The Thesis Committee for Krishnakali Sarkar
Certifies that this is the approved version of the following thesis:

ARNT isoforms differentially regulate cancer cell growth through a p53-dependent mechanism.

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:

Casey W. Wright

Haley Tucker

ARNT isoforms differentially regulate cancer cell growth through a p53-dependent mechanism.

by

Krishnakali Sarkar M.Sc.

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Dedication

Dedicated to my husband

Ayan Guha

and son

Aarav Guha

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Most importantly, I would like to thank my advisor Casey Wright for his guidance and coaching throughout the time I spend in his laboratory. I would also like to thank Haley Tucker for reading my thesis. She was always there to motivate me with her encouraging words and enlighten me with her wisdom. In addition, I also want to thank the Wright lab members: Kacie Gardella, Sunhee Lee and Israel Muro for their help, constant support and research inputs. I would specially like to thank Kacie Gardella for all the help that she has offered to me during my stay in the Laboratory and more so with her inputs while I was trying to put my thesis together. At last but not the least I would like to thank Gloria Fang from the Mills laboratory for her constant help and support. She was always there to lend a helping hand whenever I needed it.

Abstract

ARNT isoforms differentially regulate cancer cell growth through a p53-dependent mechanism.

Krishnakali Sarkar, MA

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Supervisor: Casey W. Wright

Aryl hydrocarbon receptor nuclear translocator (ARNT) is an important player in xenobiotic and hypoxic responses. In addition to this, my mentor has shown that ARNT is an integral cofactor of NF-**κ**B signaling. However, these initial observations of ARNT-mediated NF-**κ**B modulation were based on simultaneous suppression of the two ARNT isoforms, isoform 1 and 3, and therefore precluded the isolated examination of each isoform's function. We show here that lymphoid malignancies exhibit higher levels of ARNT isoform 1 compared to ARNT isoform 3. However, normal T and B lymphocytes are seen to harbor equal levels of ARNT isoform 1 and 3. We hypothesize that the increase in ARNT isoform 1 is necessary for the growth of these cancer cells as suppression of isoform 1 resulted in S-phase cell cycle arrest. These findings reveal that ARNT isoform 1 potentiates cell growth by antagonizing a p53 cell cycle inhibitory

mechanism and this further suggests that ARNT targeted therapies would benefit chemotherapy regimens.

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Chapter 1: Introduction

1.1 Background

The aryl hydrocarbon receptor nuclear translocator (ARNT, also known as hypoxia inducible factor-1β) is a ligand-dependent transcription factor. ARNT along with its partner, aryl hydrocarbon receptor (AHR), together referred to as the aryl hydrocarbon receptor complex (AHRC), binds to structurally diverse synthetic and naturally occurring chemicals including dioxins, flavonoids and tryptophan photoproducts (Denison and Nagy, 2003). The environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent known cytochrome P4501A1 inducer and ARNT, along with its binding partner AHR, have been long associated with an organism's response to various environmental contaminants like TCDD (Whitlock, 1999). ARNT is a class II bHLH-PAS transcription factor and the general heterodimerization partner for many bHLH proteins including AHR, hypoxia-inducible factor 1 and 2 (HIF-1α and 2α), and single-minded protein 1 and 2 (SIM1 and 2) (McIntosh et al., 2010). AHR-ARNT heterodimers regulate genes involved in the metabolism of xenobiotics (Hankinson, 1994), whereas ARNT-HIF-1α heterodimers regulate genes involved in the response to oxygen deprivation (Bunn and Poyton, 1996).

1.2 Structure of ARNT

ARNT is a member of the **basic helix-loop-helix** Per/AHR/ARNT/Sim (**bHLH-PAS) family** of transcription factors. The amino (N) terminal end of ARNT contains the bHLH-PAS domain, and the ligand-binding domains (Fukunaga et al., 1995). This

portion also includes the nuclear localization signal (NLS) and appears to have two nuclear export signals (NES) (Ikuta et al., 1998). The HLH domain is responsible for dimerization with a homologous or heterologous partner. The adjacent basic region mediates sequence specific binding. The structural analysis of the cloned cDNA revealed that the ARNT sequence contains a conserved region called PAS domain shared by *Drosophila* Per, Ah receptor (AHR), and *Drosophila* Sim (Hoffman et al., 1991). The PAS domain has about 260 amino acids containing two short stretches of internal direct repeats called PAS A and PAS B (Nambu et al., 1991). The PAS domains of Per and Sim mediate heterodimerization between these proteins, whereas the PAS domain of Per can also mediate homodimerization (Huang et al., 2013). The carboxy (C) terminal end of ARNT contains a transactivation domain (TAD), which has three subdomains, the acidic, glutamine-rich, and proline-serine-threonine rich domains (Ma et al., 1995). The TA domain is the key determinant of cellular localization and ligand-independent nucleocytoplasmic shuttling properties (Ramadoss and Perdew, 2005). Once the mouse ARNT (Reisz-Porszasz et al., 1994) and human AHR (Itoh and T, 1993) were cloned, it was evident that the ARNT and AHR of both the species were about 20% identical to each other in their amino acid sequence and had a striking resemblance in their overall structure (Hankinson, 1994). The ARNT gene is found to be located on human Chromosome 1q21 and on mouse Chromosome 3 near Cf-3 (Doedens et al., 2013) whereas AHR is located on human chromosome 7 (Le Beau et al., 1994) and mouse chromosome 12 (Poland et al., 1987).

ARNT is expressed as two alternatively spliced isoforms, ARNT isoform 1 and ARNT isoform 3 in humans, or Arnt-a and Arnt-b in mice. ARNT Isoform 1 has 789 amino acids and isoform 3 has 774 amino acids, thus differing in 15 amino acids found near the N-terminus of isoform 1 (Hoffman et al., 1991) (Figure 1.1). These unique 15 amino acids in isoform 1 are mainly due to the inclusion of exon 5 by the splice machinery. Interestingly, the amino acids encoded by exon 5 are directly upstream of the bHLH DNA binding motif and the last 2 carboxyl amino acids contribute to DNA interactions (Sun et al., 1997). In ARNT isoform 3 where exon 5 is missing, the junctions come together to reiterate the start of the bHLH DNA binding motif. This provides an extra phosphorylation site on exon 5, a serine 77, which has been shown to have a profound affect on ARNT isoform 1 DNA binding activity. Initial work from our laboratory has shown that equal amounts of ARNT isoforms are present in a number of human cell types like the T and the B lymphocytes, hepatocytes and epithelial cells (unpublished data). Based on our initial data we hypothesize that the expression of ARNT isoforms is different in malignant cell lines and different ARNT isoforms differentially regulate cancer growth.

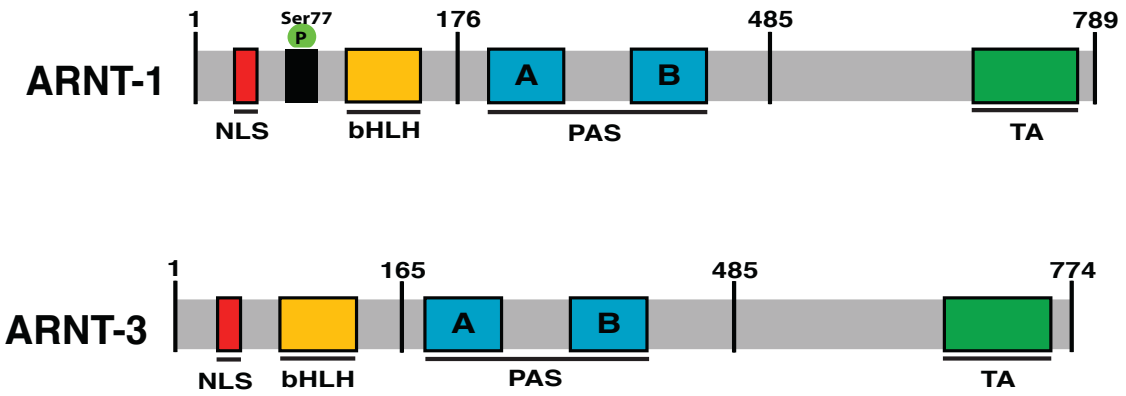


Figure 1.1: **Schematic representation of ARNT isoform 1 and 3.**

The N terminal has the bHLH-PAS domain, the C terminal end has the TA domain. ARNT Isoform 1 has 789 amino acids and isoform 3 has 774 amino acids, thus differing in 15 amino acids found near the N- terminus of isoform 1.

1.3 The AHR/ARNT Signaling Pathway

Many endogenous and exogenous chemicals possess the ability to bind to and activate AHR. Endogenous compounds that are thought to activate the AHR are bilirubin or various tryptophan metabolites. However, the majority of the known AHR activating compounds are synthetic in nature. Classical chemical compounds that activate the AHR include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (B(a)P), 3-methylcholanthrene (3MC), β-naphtoflavone and the dioxin-like polychlorinated biphenyls (PCB) which have a coplanar conformation that allows binding to the AHR (Denison and Nagy, 2003) . AHR target genes include phase I detoxifying enzymes such as the cytochrome P450 (CYP) 1 family members as well as phase II detoxifying enzymes such as aldehyde dehydrogenase 3, UDP-glucuronosyl transferase, and glutathione S-transferase (Beischlag et al., 2008).

In its nonligand state, the AHR resides in the cytoplasm in an inactive form bound to a chaperone protein complex that includes heat shock protein 90 (McGuire et al., 1994b), AHR-interacting protein (AIP), and p23 (Hankinson, 1994) (Nair et al., 1996). Once activated by a ligand, AHR translocates into the nucleus where it is released from the complex and then it associates with its heterodimerization partner ARNT (McGuire et al., 1994a). The AHR/ARNT heterodimer then binds to its cognate DNA sequence motif, referred to as the dioxin-responsive element (DRE), or AHR-responsive element (AHRE; 5′-TNGCGTG-3′) with a minimum core sequence of 5′-GCGTG-3′ upstream of a responsive gene such as cytochrome P450 1A1 (CYP1A1) and other AHR-dependent–responsive genes. This leads to DNA bending, recruitment of coactivators, chromatin and

nucleosome disruption, increased promoter accessibility to transcription factors, and increased rates of gene transcription (Beischlag et al., 2008), (Swanson, 2002).

Once bound to chromatin, the activated AHR/ARNT heterodimer induces the recruitment of coregulator proteins resulting in changes in the target gene expression, including CYP1A1, CYP1B1, nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; NRF2), and AHRR (Whitlock, 1999), (Zhang et al., 2013). AHR/ARNT heterodimers also recruits components of the SWI/SNF complex that remodel chromatin, coactivators such as the steroid receptor coactivator-1 (SRC-1) with histone acetyltransferase (HAT) activity, and components of the positive transcription elongation factor (P-TEFb) (Tian et al., 2003) to control activity or the general transcription machinery (GTM).

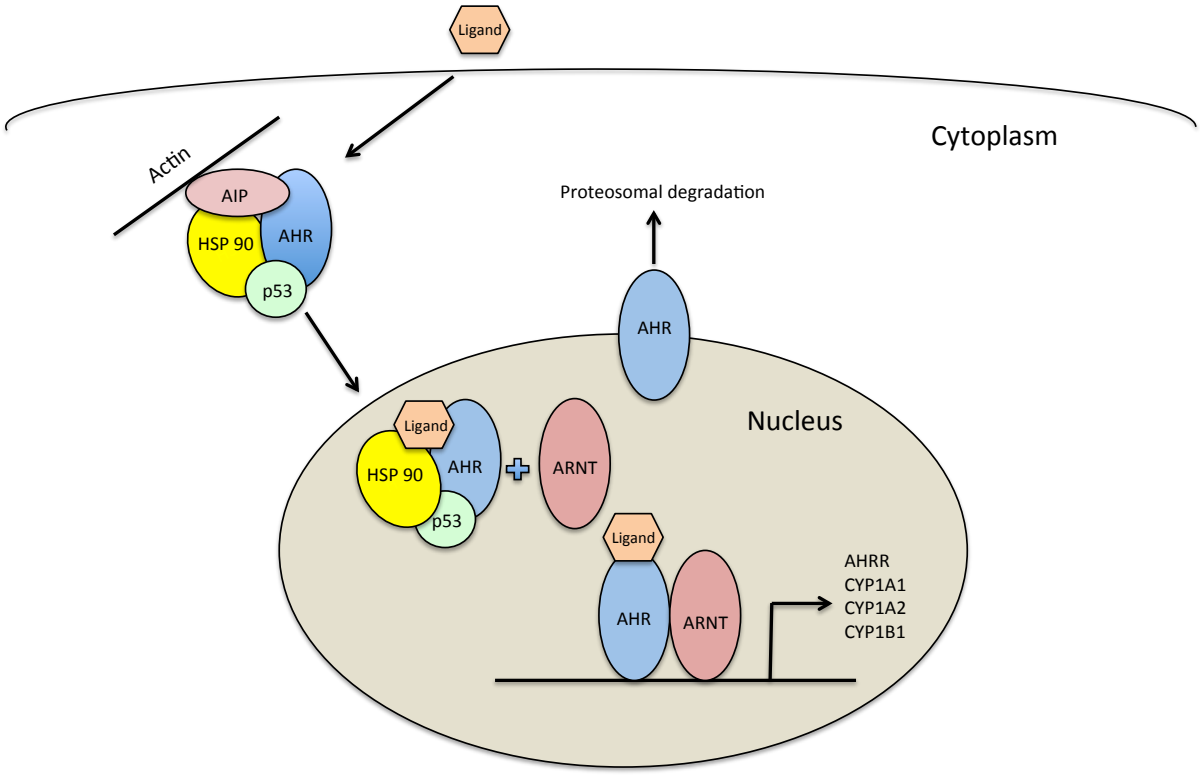


Figure 1.2: **The AHR/ARNT signaling pathway.**

Adapted from Brigitta Stockinger Annu. Rev. Immunol. 2014.

In the absence of a ligand, AHR is present in the cytoplasm bound as an inactive complex with several chaperone proteins, including HSP90, AIP, and p23. Upon ligand binding, it translocates into the nucleus, where it is released from the complex, heterodimerizes with its protein partner ARNT, and finally binds genomic regions containing its binding motif [dioxin response element (DRE)], inducing transcription of target genes such as CYP1A1, CYP1A2, CYP1B1, and AHR represor (AHRR). AHR signaling is regulated at three levels: proteasomal degradation of AHR, ligand metabolism by CYP1A1, and AHR/ARNT complex disruption by AHRR.

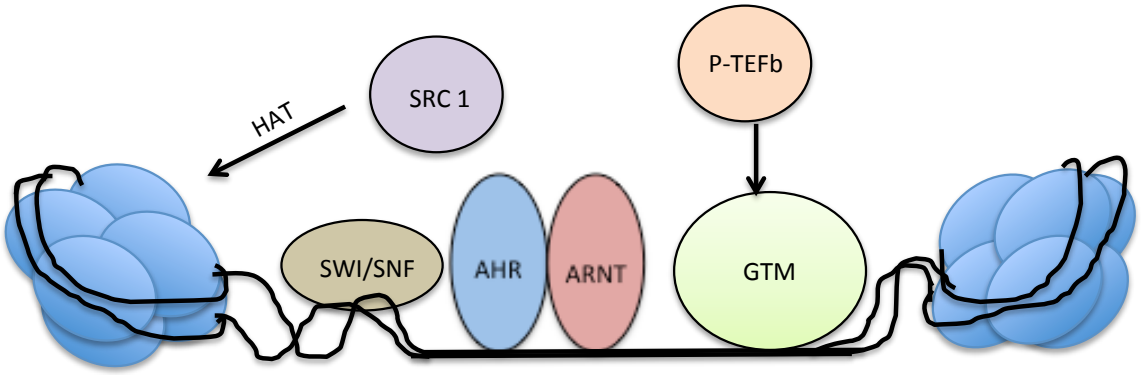


Figure 1.3: **AHR/ARNT heterodimers recruits components of the SWI/SNF complex.**

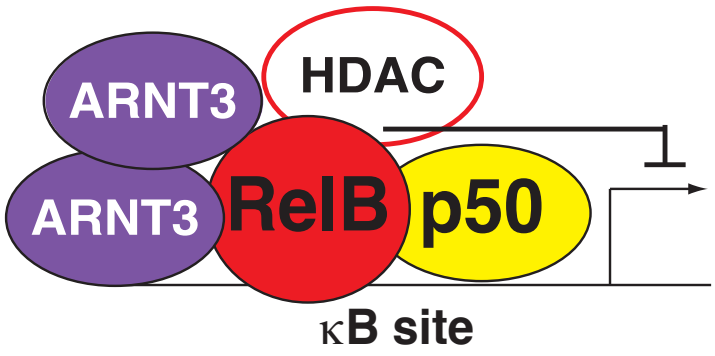
Adapted from Brigitta Stockinger Annu. Rev. Immunol. 2014.

AHR/ARNT heterodimers recruit multiple protein partners to regulate transcriptional activation of their target genes. These include components of the SWI/SNF complex that remodel chromatin, coactivators such as the steroid receptor coactivator-1 (SRC-1) with histone acetyltransferase (HAT) activity, and components of the positive transcription elongation factor (P-TEFb) to control activity or the general transcription machinery (GTM).

1.4 Regulatory involvement of ARNT in NF-κB signaling

The pleiotropic transcription factor, nuclear factor-κB (NF-κB), is essential in the regulation of innate and adaptive immunity (Staudt, 2010). NF-κB controls inflammation by regulation of gene transcription, which is achieved through differential dimerization of five subunits: Rel-A, Rel-B, c-Rel, p105/p50, and p100/p5 (Ghosh and Hayden, 2012). The subunits homodimerize or heterodimerize through a common rel homology domain (RHD) to form transcriptionally active dimers. Prior to receiving a signal, NF-κB dimers are kept inactive in the cytoplasm by binding the inhibitors of NF-κB (IκB). Once the NF-κB pathway is initiated, a signaling cascade causes the ubiquitination and proteasomal degradation of the IkB proteins, allowing the dimers to translocate to the nucleus to regulate gene transcription. There are two NF-κB pathways that are defined by the dimer that gets released: canonical and noncanonical (alternative) pathways. The canonical pathway is characterized by degradation of IκBα followed by the release of RelA-p50 dimers; while, in noncanonical signaling, processing of p100 to p52 releases RelB-p52 dimers (Hayden and Ghosh, 2012), (Sun, 2012). NF-κB requires a number of negative regulatory mechanisms to prevent excess transcriptional activity that can lead to chronic inflammation, autoimmune diseases, and cancer (Ruland, 2011). One example is the subunit RelB. RelB was originally identified as an inhibitor of the subunit p50. RelB negatively regulates the canonical NF-κB pathway, most likely through its association with p50 (Ruben et al., 1992). My mentor in his previous studies discovered that ARNT binds to RelB and has a negative regulatory effect on NF-κB signaling. RelB is present at classical RelA-p50 regulated target promoters. Upon initial signaling, RelA-p50

translocates to the nucleus where it replaces the RelB complex. Once stimulated, RelB translocates to the nucleus where it associates with ARNT thus enhancing the binding of RelB to DNA and negatively regulating RelA-p50complexes (Wright et al., 2007). However, AHR and ARNT are also found to differentially and independently regulate NF-κB signaling and chemokine CXCL8 (IL8), CCL5 (RANTES) responses induced by both inhaled pollutants and pulmonary infection. Overik J *et al*. propose that AHR signaling may be a weak activator of p65 signaling that suppresses p65 activity induced by strong activators of NF-κB (Ovrevik et al., 2014).



Adapted from Wright C.W. Science (2009)

Figure 1.4: **Proposed model of ARNT regulation of NF-κB activity.**

Aryl hydrocarbon receptor nuclear translocator (ARNT) binds to RelB to promote higher affinity of RelB DNA binding. Consecutively, RelB has a negative regulatory effect on canonical NF-κB signaling. Before stimulation RelB is present at classical RelA-p50 regulated target promoters.

1.5 The role of AHR in NF-κB signaling

The AHR is a cytoplasmic receptor for xenobiotic ligands. For decades AHR has been recognized as the key mediator of xenobiotic responses to environmental pollutants. These pollutants include polycyclic aromatic hydrocarbons like [B(A)P] and halogenated hydrocarbons such as TCDD, one of the most potent activators of AHR (Denison and Nagy, 2003). The AHR and NF-κB are both inducible transcription factors, each governing the expression of distinct sets of genes that are important for the normal physiology as well as for pathophysiological responses. Recent reports suggest that AHR affects the expression and function of many immunoregulatory genes and also has an influence on differentiation of inflammatory dendritic cells (Bankoti et al., 2010). Furthermore, AHR also plays a critical role in T-cell differentiation and immunity of the gut (Quintana et al., 2008), (Veldhoen et al., 2008). Denison *et al.* in their recent study has dissected the molecular mechanisms of AHR activity in immune cells during inflammation (Vogel et al., 2014b). NF-κB is a key transcription factor in regulating the immune system and inflammatory responses. AHR has gradually emerged as a regulator of inflammation in various tissues. There seems to be a crosstalk between the AHR and NF-κB family of transcription factors (Tian et al., 2002), (Vogel et al., 2014a). In their present study, Denison *et al.* gave an insight into the mechanism of the regulation of the human AHR gene through NF-κB signaling. Elevated expressions of AHR-regulated genes were documented upon LPS stimulation. LPS activated NF-κB signaling, in a RelA-dependent manner, which in turn increased the expression of AHR and CYP1A1

(Vogel et al., 2014a). This signifies that inflammatory stimuli and cytokines that regulate NF-κB induce AHR expression during activation and differentiation of immune cells.

1.6 p53 "The guardian of the genome"

p53 is an intensively studied protein, its importance is mainly due to the role it plays as a tumor suppressor in humans and other mammals. The p53 gene is the most frequently mutated gene in human cancers, about 50% of all human cancers have a mutated p53 or an inactive version of p53 (Toledo and Wahl, 2006). In brief, p53 is a master transcription factor that under normal conditions is usually functionally inactive due to its rapid degradation by the ubiquitin ligase MDM2. However, upon the infliction of almost literally any cellular stress, MDM2-driven degradation is jeopardized, and p53 accumulates and gains full control in transcriptional activation (Hayon and Haupt, 2002). The p53 transcriptional cascade includes the activation of a number of cell cycle inhibitors and pro-apoptotic proteins, which results in apoptosis or irreversible proliferative arrest, also known as senescence (Vousden and Lu, 2002). Two types of stresses that are targeted to p53 are DNA damage and oncogenic signaling. DNA damage was the first type of stress found to activate p53 and based on this, p53 has been widely and rightly regarded as "the guardian of the genome" (Chao et al., 2000). The other factor that is found to activate p53 is oncogenic signaling. Therefore, analogous to the title "guardian of the genome", p53 is also assigned the function of "policeman of the oncogenes"(Efeyan and Serrano, 2007). Briefly, oncogenic signaling activates p53 through ARF (Palmero et al., 1998), which interacts with MDM2 inhibiting its p53-

ubiquitin ligase activity. In this manner, ARF-dependent stabilization of p53 results in a dramatic increase in p53 activity. Further experiments by Efeyan et al demonstrated that DNA damage response of p53 is of minor importance for cancer protection and this tumor protective activity of p53 was mainly dependent on the presence of ARF, as the oncogenic signaling mediated activation of p53 was completely lost in the absence of ARF in ARF-null mice (Efeyan and Serrano, 2007). Thus, it is well documented that activation of p53 occurs in response to a number of cellular stresses, including DNA damage which eventually leads to the activation of several genes whose products trigger cell cycle arrest, apoptosis, or DNA repair (Lakin and Jackson, 1999), (Laptenko and Prives, 2006).

Experiments in our laboratory with the suppression of ARNT isoform 1 using siRNA, resulted in cell cycle arrest. Since, p53 is the key regulator of cell cycle arrest, this guided us to postulate that p53 was involved in this process. We further wanted to explore whether changes in ARNT expression would have any effect on p53 activation in malignant lymphoid cells.

Chapter 2: Experimental Design and Methods

2.1 Cell culture and Reagents:

Karpas 299 cells were cultured in RPMI-1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Atlas Biologicals, Inc.) and 2 mM Glutamax (Life Technologies) at 37 °C and 5% CO_2. For transfections, cells were recovered in RPMI-1640 supplemented with 20% fetal bovine serum and 2 mM Glutamax. Human T and B lymphocytes were isolated using the MACS Miltenyi Biotec CD4 T Cell Isolation Kit. Unless otherwise stated, all reagents and chemicals were obtained from Sigma- Aldrich (St. Louis, MO)

2.2 RNA interference

Cells were transfected by electroporation as described (Wright 2007). Briefly, 10×10^6 cells (at 25×10^6 cells/mL) were electroporated using a Gene Pulsar Xcell (Bio-Rad) with 2 μM control, ARNT isoform 1 or ARNT isoform 1 and 3 target siRNA duplexes. Immediately after electroporation, cells were placed in recovery media (20% serum) for 16 hours and then transferred to growth media (10% serum) at 0.5 x 10^6/ mL for an additional 24 hours. Control, ARNT isoform 1and ARNT isoform 3 siRNA duplexes were obtained from Sigma-Aldrich.

Target sequences are listed in Table 2.1:

Control- sense	5’-CAUGCCUUGCUUUACGCAUTT-3’
Control- antisense	5’-AUGCGUAAAGCAAGGCAUGTT-3’
ARNT isoform1- sense	5’-UGCCAGGUCGGAUGAUGAGCA-3’
ARNT isoform1- antisense	5’- UGCUCAUCAUCCGACCUGGCA-3’
ARNT isoform1/3 sense	5’-UCCAGUCUCAGGAGCAAAG-3’
ARNT isoform1/3 antisense	5’-AAACUGGGAAGUACGAGUC-3’

Table 2.1: siRNA target sequences.

2.3 Antibodies and Immunoblotting

After electroporation the cells were harvested. Lysates from cells were prepared by incubating cells on ice in radioimmune precipitation (RIPA) buffer (PBS containing 1% Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% SDS, 1 mM PMSF, and 1 mM DTT) supplemented with complete mini protease inhibitor tablets (Roche Diagnostics). The cells were then incubated on ice for 20 minutes and centrifuged at 4 $^{\circ}$C at 21,000 x g for 10 minutes. Protein lysates were then resolved on denaturing polyacrylamide gels, transferred to nitrocellulose (Whatman) membrane, and blocked with 5% powdered milk (w/v) in Tris-buffered saline with Tween 20. (TBS-T; 20mM Tris-HCL, pH 7.6, 137mM NaCl containing 0.1% Tween 20). The membranes were incubated with the following primary antibodies, washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Peroxidase activity was then detected by the

enhanced chemiluminescence Western blot analysis system (GE Healthcare). Antibodies used are: β-Actin (Sigma-Aldrich), ARNT (BD Biosciences), IκB-α (Millipore), p53 (Invitrogen), phosphorylated $p53^{S15}$(R & D systems).

2.4 Cell cycle analysis

After 48 hours of siRNA treatment cells were harvested and washed with PBS. Cells were fixed at a concentration of 1×10^6 in 1 mL of 50% ethanol in PBS for 1 hour at -20 °C. The ethanol was then removed from the cells and then placed in PBS containing 50 μg/mL of propidium iodide and 100 μg/mL of RNAse A. Flow cytometry was performed using the Beckman Coulter Cytomics FC500 and data analysis was performed using FlowJo (Tree Star Inc.).

2.5 Isolation of T and B-lymphocytes

Freshly drawn normal leukocytes were obtained from The New York Blood Center with proper IRB certification. Human peripheral blood mononuclear cells (PBMC) from the leukocytes were isolated by Ficoll centrifugation at 500 x g for 30 minutes. The cells in the buffy layer are washed with PBS and cultured overnight in RPMI 1640 media. Using the MACS Miltenyi Biotec CD4 T Cell Isolation Kit, human T lymphocytes cells are isolated by depletion of non-target cells (negative selection). Non-target cells are labeled with a cocktail of biotin-conjugated monoclonal antibodies and the CD4 T Cell Micro Bead Cocktail. The magnetically labeled non-target T cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the

unlabeled T helper cells pass through the column. Similarly, using the MACS Miltenyi Biotec B Cell Isolation Kit II, human B cells are isolated by depletion of non-B cells (negative selection). Non-B cells are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to Micro Beads, as secondary labeling reagent. The magnetically labeled non-B cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled B cells pass through the column.

2.6 Statistics

Statistical significance was evaluation by using the student's t-test. A P value of $p<0.05$ was considered statistically significant.

Chapter 3: Results and Discussion

3.1 ARNT levels in normal T cells, B cells and Karpas 299 cells

To study the ARNT levels in lymphoid malignancies, lysates from the T cell derived anaplastic large cell lymphoma (ALCL) cell line, Karpas 299, were subjected to western blot analysis with ARNT antibody. In addition we examined ARNT isoform expression in human T and B lymphocytes. Immunoblot analysis of the lysates showed high levels of ARNT isoform 1 as compared to ARNT isoform 3 in Karpas 299 cells (Figure 3.1). This result is consistent with previous results from our laboratory showing an increase in ARNT isoform 1 levels in various other lymphoid malignancies. Our laboratory for the first time has shown that this increase in ARNT isoform 1 levels is mainly necessary for the growth of ALCL and multiple myeloma (MM) cancer cell lines. However, naive human T and B lymphocytes expressed equal amounts of both isoforms with T cells displaying higher ARNT expression than B cells.

3.2 ARNT isoforms differentially regulates p53 activity

Data from our laboratory have demonstrated that suppression of ARNT isoform 1 leads to changes in the expression of cell cycle genes like p21 and p15. It is well known that p53 is one of the key regulators of cell cycle arrest. Here, we sought to extend these findings by determining whether changes in the expression of ARNT isoforms will impact p53 activity, a key regulator of cell cycle arrest. To dissect the involvement of ARNT in regulating p53 activity, we used ARNT isoform specific siRNA to suppress isoform 1 (siARNT-1) or siRNA that targeted both isoforms (siARNT-1/3). Karpas 299

cells were transfected with the indicated siRNA duplexes for 48 hours followed by western blot analysis to determine phospho and total p53 levels as well as ARNT isoform 1 and 3 levels. The siRNA used in this experiment were effective in reducing ARNT isoform 1 and 3 protein levels as shown in Figure 3.2 panel C. Interestingly, our results indicate that supression of ARNT isoform 1 leads to an increased phosphorylation of p53 indicative of p53 activation. Conversely, suppression of both ARNT isoforms 1 and 3 leads to decreased phosphorylation of p53 (Figure 3.2 Panel A), most likely linked to the fact that we observed a concomitant decrease in total p53 levels upon suppression of both ARNT isoforms 1 and 3. The stabilization and activation of p53 in our system was measured by the phosphorylation of serine 15 of p53. (Figure 3.2 Panel B). Panel D shows the confirmation of the ARNT isoform specific RNAi.

3.3 Cell cycle profile of Karpas 299 cells transfected with the indicated siRNA duplexes.

Based on our results, we hypothesized that transfection of Karpas 299 cells with siRNA targeting ARNT isoform 1 will lead to cell cycle arrest due to increased activation of p53, whereas transfection of siRNAs targeting both ARNT isoforms will not affect cell cycle progression. To study the cell cycle profile, Karpas 299 cells were transfected with the indicated siRNA duplex (Figure 3.3). Our results indicate that suppression of ARNT isoform 1 reduces cell growth in this malignant lymphoid cell line due to a S-phase cell cycle arrest. Flow cytometric analysis indicated that the number of cells arrested in the S phase was higher in cells transfected with siRNA against ARNT isoform 1 in comparison to those transfected with control siRNA or siRNA targeting ARNT isoform 1 and 3.

These results provide functional evidence for increased p53 activation by the suppression of only ARNT isoform 1 in Karpas 299 cells, thus suggesting that ARNT isoform 3 is mediating p53 activation. Further, preliminary data from our laboratory has shown that this cell cycle arrest correlates with increased expression of cell cycle inhibitory genes like p15 and p21.

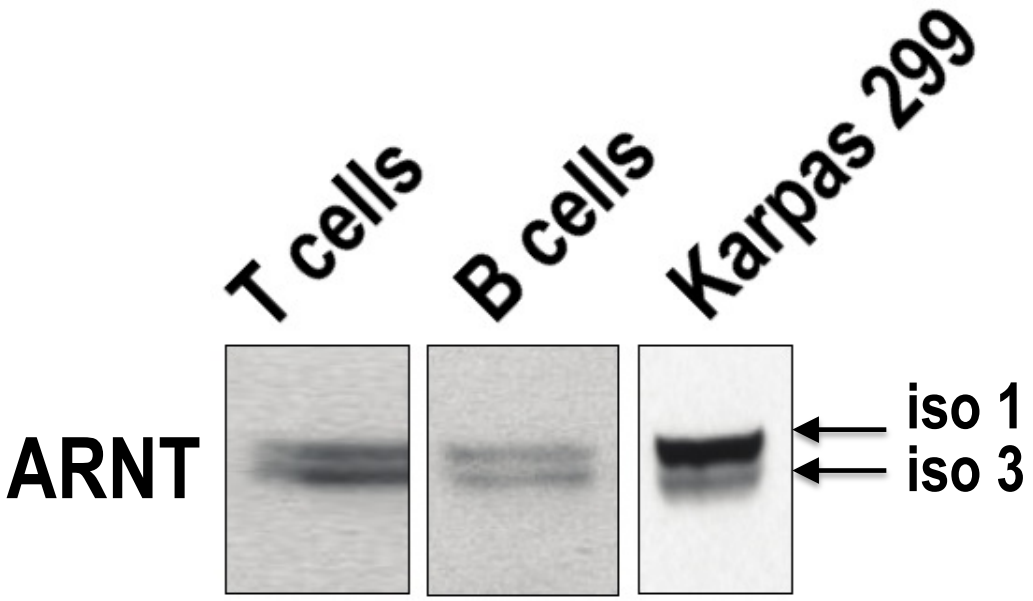


Figure 3.1: **ARNT levels in normal T cells, B cells and Karpas 299 cells.**

The ARNT isoform ratio is equal in normal lymphocytes. T cells express more ARNT as compared to B cells. T and B cells were isolated from healthy human donors, lysed and analyzed by Western blotting with anti ARNT. For comparison the T cell derived ALCL cell line Karpas 299 were run alongside T and B cells.

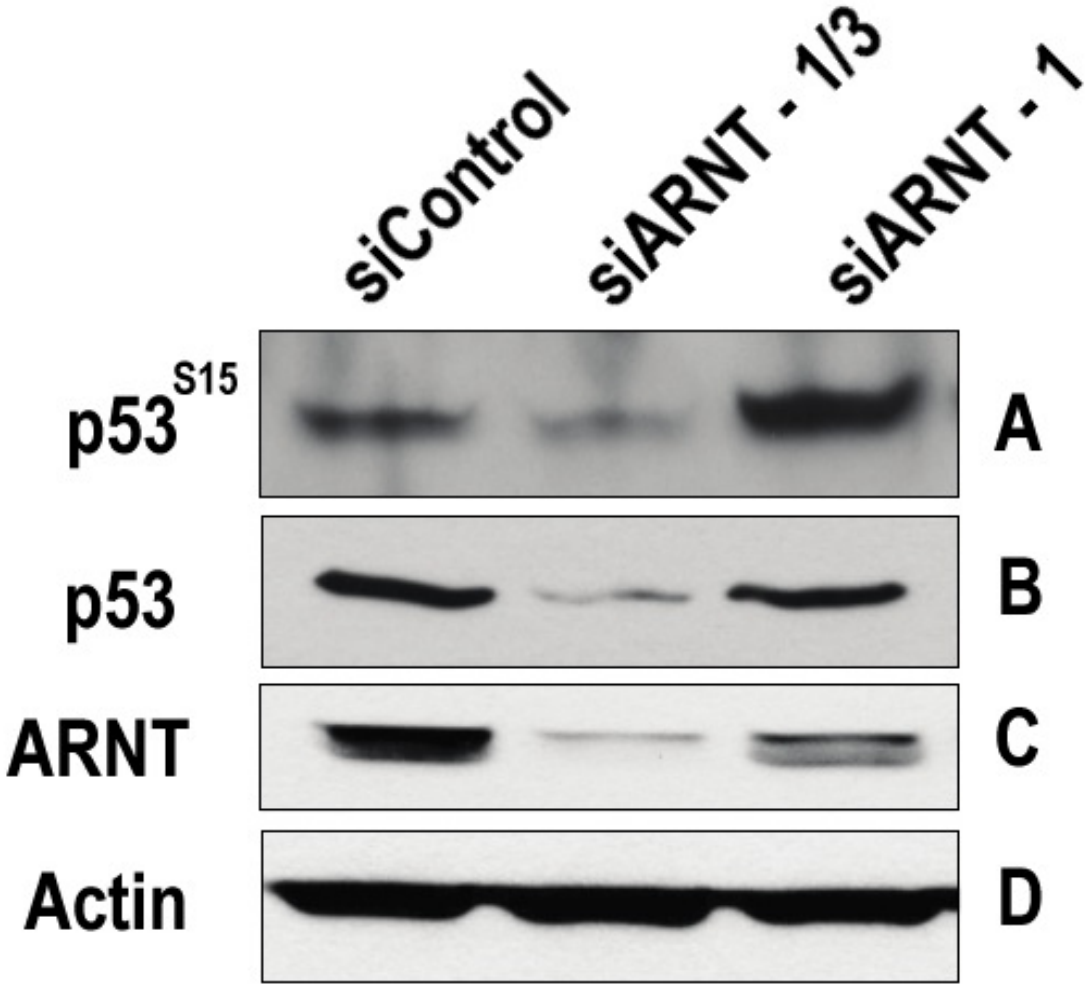


Figure 3.2: **ARNT isoforms differentially regulates p53 activity.**

Cells were transfected with control scrambled siRNA or si RNA directed against ARNT isoform 1 and 3. Equal amount of proteins were loaded, protein levels of p53, $p53^{S15}$ and ARNT were detected by western blotting as described in experimental design and methods.

Panel A: Stabilization and activation of p53 as measured by phosphorylation of serine 15

Panel B: p53 is stabilized and activated in the nucleus with suppressed ARNT-1

Panel C: Confirmation of the ARNT isoform specific RNAi.

Panel D: Actin was used as a control for protein loading.

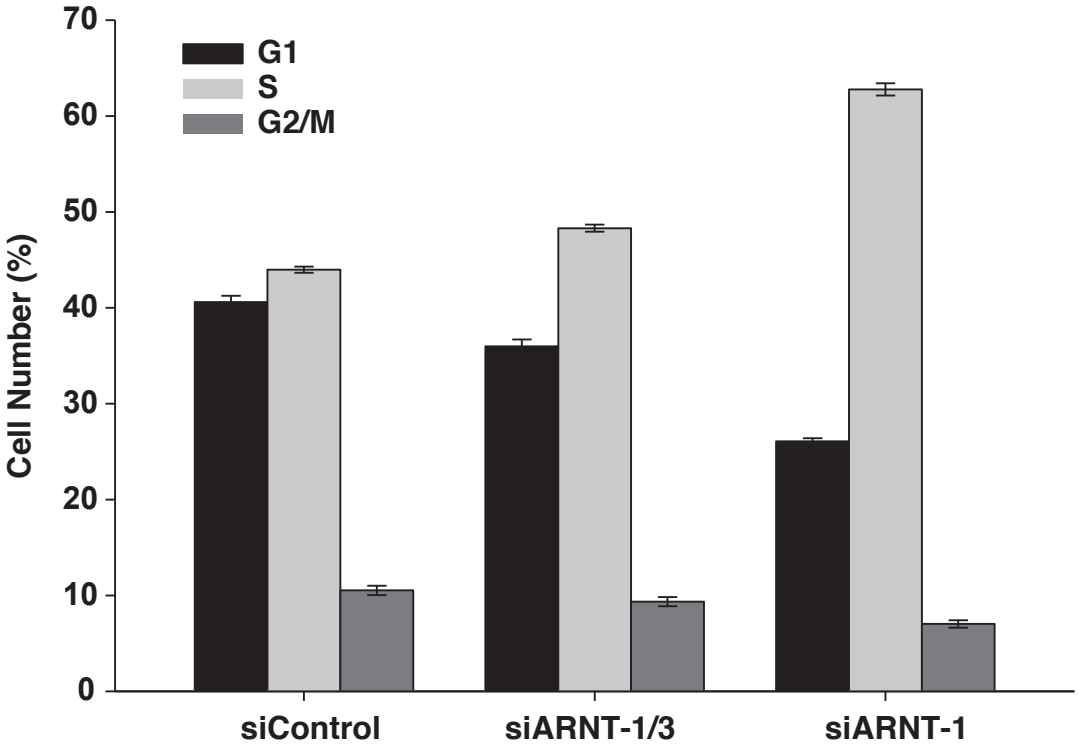


Figure 3.3**: Flow cytometry was conducted to assess the cell cycle profile.**

ALCL (Karpas 299) cells were transfected with either siRNA control (C) or ARNT isoform 1 or 3 for 48 hours and then stained with propidium iodide (PI) to examine the cell cycle profile by flow cytometry.

Suppression of ARNT isoform 1 reduces cell growth in this malignant lymphoid cell line due to a S-phase cell cycle arrest.

3.4 Discussion:

Genetic mutations including deletions, chromosomal translocations, transitions and amplifications are a common phenomenon in lymphoid malignancies. Cancer cells often produce multiple gene copies of proteins that promote proliferation and survival. Many of the isoforms produced in this manner are developmentally regulated and are preferentially re-expressed in tumors (David CJ). Earlier data from our laboratory has shown that ARNT is expressed as two alternatively spliced isoforms in most, but not all, cell types including hepatocytes, epithelial cells, fibroblasts and lymphocytes. Interestingly, ARNT is found to be the most highly amplified gene observed in certain T cell lymphoma, MM tumors and malignant plasma B cell lines established from MM patients (Bergsagel L, personal communications). Work from our laboratory has also demonstrated an increase in ARNT isoform 1 in various lymphoid malignancies. Earlier data from our laboratory clearly showed high levels of ARNT isoform 1 as compared to ARNT isoform 3 in Karpas 299 cells. Expression of multiple gene copies of ARNT may be responsible for disrupting the alternative splicing machinery (Faustino NA), as a result of which there is an increased presence of ARNT isoform 1. Thus, we have now shown that increased ARNT isoform 1 promotes the growth of lymphoid malignancies.

Our data shows that suppression of ARNT isoform 1 results in increased expression of p15 and p21, genes that are involved in cell cycle arrest. Since p53 is the key regulator of cell cycle arrest, we wanted to determine whether decreases in ARNT isoform 1 protein would affect p53 stability and/or activity in Karpas 299 cells. Indeed, our data demonstrates for the first time that p53 is stabilized and activated in Karpas 299

cells after suppression of ARNT isoform 1. However, in contrast to our findings where ARNT isoform 1 was suppressed, simultaneous suppression of both ARNT isoforms 1 and 3 leads to a decrease in phosphorylated p53. More importantly, this leads to an overall reduction in p53 protein levels. One possible interpretation of this result is that ARNT isoform 1 functions to suppress ARNT isoform 3. Hence, suppression of ARNT isoform 1 relieves ARNT isoform 3, and leads to enhance p53 activation. Our data reveals a very stable p53 after suppression of ARNT isoform 1 but not so after suppressing both the ARNT isoforms.

Analysis of the cell cycle profile of Karpas 299 cells transfected with the ARNT siRNA duplexes revealed that suppression of ARNT isoform 1 resulted in a S-phase cell cycle arrest. Thus we can conclude that an increase in ARNT isoform 1 in Karpas 299 cells is necessary for the growth of ALCL cancer cells since the suppression of ARNT isoform 1 resulted in S-phase cell cycle arrest. Based on this data along with other data from our laboratory we propose a working model to explain the effects of ARNT on cell cycle progression (Figure 3.4). According to our model, ARNT isoform 1 is inhibitory and so suppressing ARNT isoform 1 releases ARNT isoform 3 and RelB. There are three possible mechanism of action, ARNT isoform 3 and RelB can directly regulate cell cycle genes or can regulate p53, which in turn regulates cell cycle genes. It may also regulate cytokine macrophage inhibitory cytokine 1 (MIC 1) that feedback in an autocrine manner to activate p53 and induce cell cycle arrest. So, ARNT has some effect directly or indirectly on the cytokine expression through modulating p53 and cell cycle genes.

Our future goal is to define the mechanism of ARNT assisted cancer cell growth. Importantly, the fact that an increase in ARNT isoform 1 levels, in lymphoid malignancies, gives a growth advantage suggesting that ARNT isoform 1 would be a good target for therapeutic intervention. Therefore, our long-term goal is to elucidate the role of ARNT in regulating key immune signaling pathways, which will be the stepping-stone to the development of not only anticancer therapies but also therapies against various other autoimmune diseases.

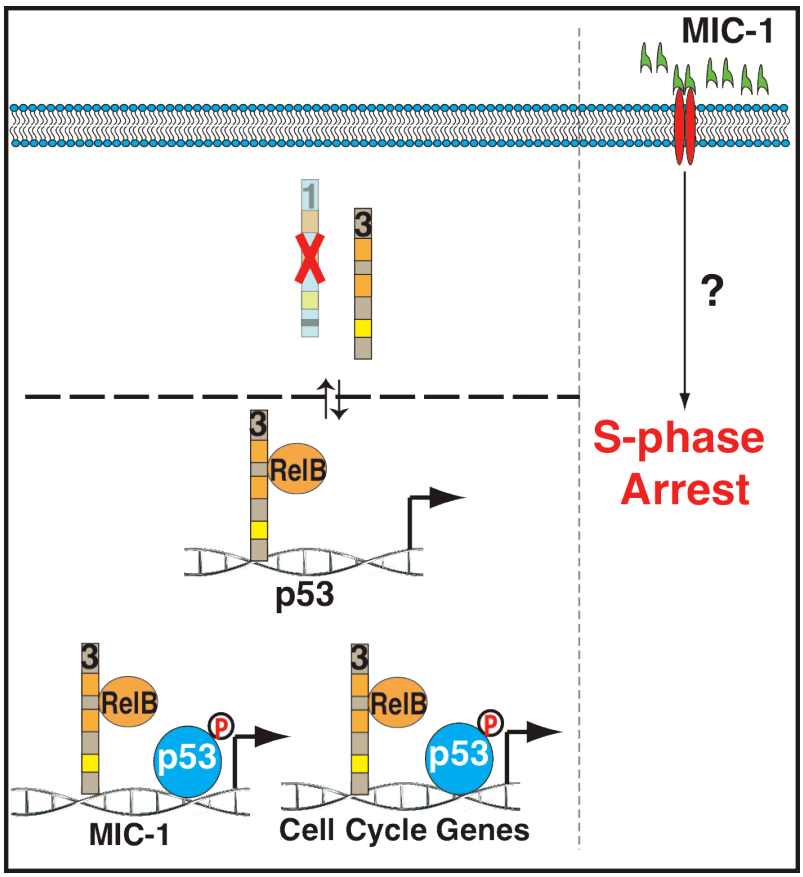


Figure 3.4: **Working hypothetical model**

ARNT isoform 1 is inhibitory and so suppressing ARNT isoform 1 releases ARNT isoform 3 and RelB. There are three possible mechanism of action, ARNT isoform 3 and RelB can directly regulate cell cycle genes or can regulate p53, which in turn regulates cell cycle genes. It may also regulate cytokine macrophage inhibitory cytokine 1 (MIC 1) that feedback in an autocrine manner to activate p53 and induce cell cycle arrest. So, ARNT has some effect directly or indirectly on the cytokine expression through modulating p53 and cell cycle genes.

Chapter 4: Regulation of alcohol consumption by inflammation.

One of my other projects was concerned with the influence of NF-κB in the brain. More importantly, we wanted to analyze the effect of neuro-inflammation on inducing alcohol consumption. As such unmitigated inflammation is an important contributor to disease in a variety of tissues, and interestingly, has been implicated as a contributing factor to alcoholism. However, it is unclear exactly which cell types namely microglia or astrocytes contribute to the neuro-inflammation that facilitates alcohol consumption. We were mainly interested in looking at the effects of alcohol on brain microglia.

4.1 Introduction:

Microglia are the resident macrophage like cells in the brain that paly a major role in the host defense and tissue repair in the central nervous system (CNS) (Kreutzbe, 1996). Under pathological conditions, activated microglia release neurotoxic and pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), reactive oxygen species and pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Meda et al., 1995), (Dandona et al., 2010). Overproduction of these inflammatory mediators and cytokines causes severe neurodegenerative diseases, including Alzheimer's disease (Perlmutter et al., 1990), Parkinson's disease, cerebral ischemia, multiple sclerosis (Banati and Graeber, 1994) and trauma (Dheen et al., 2007). Activated microglia are a major source of pro-inflammatory and/or cytotoxic factors that cause neuronal damage in the CNS (Eikelenboom and van Gool, 2004). Previous studies have also demonstrated that a decrease in the number of pro-inflammatory mediators in microglia may attenuate the severity of these disorders

(Gao et al., 2003) and (Liu and Hong, 2003). Hence, microglia cells are extensively studied to access brain inflammation. However, for a typical preparation of rodent microglia a number of brains are required to yield cells for a limited amount of experiments on signaling and disease mechanism. To overcome this limitation most of the microglial activation and signaling are performed in vitro, using BV2 cells. BV2 cells were derived from raf/myc immortalized murine neonatal microglia (Blasi et al., 1990) and are the most frequently used substitute for primary microglia. As shown by Anja Henn et al., the overall response pattern of BV2 cells was parallel to that of primary microglia (Henn et al., 2009).

Studies of peripheral macrophages have led to the concept of different macrophage activation states, ranging from 'classical' activation (also referred to as M1-type macrophage activation) to so-called 'alternative' activation (also referred to as M2-type macrophage activation). Classically activated microglia initiate T cell response. Activated microglia turn on MHC class II expression, which is required for activation of naive T cells, and produce numerous pro-inflammatory cytokines, including cytokines that induce the differentiation of effector T cells. Since microglia cells are related to monocytes and macrophages, they can be readily activated by cell wall components of bacteria such as lipopolysaccharides (LPS). It is possible that injections of LPS initiate signaling through macrophages that are highly enriched in the peritoneal cavity; LPS may also diffuse into the bloodstream, where it stimulates circulating immune cells (1996). Toll-like receptor 4 (TLR4) is a pattern recognition receptor that binds to pathogen-associated molecular patterns in LPS and initiates a signaling cascade through nuclear

factor κB (NF-κB)-dependent and NF-κB-independent pathways (Palsson-McDermott and O'Ne, 2004). The peripheral LPS signal permeates the CNS and activates resident microglia within the brain (Glezer et al., 2003). However, it remains to be determined whether peripherally injected low doses of LPS directly stimulate microglia or whether they induce microglial activation through downstream surrogate messengers that are produced by immune cells or brain microvascular endothelial cells. Upon activation, microglia can become phagocytic and secrete a multitude of noxious cytokines, chemokines, and oxygen radicals. Activated microglia can also perform neuroprotective functions (Ransohoff and Perry, 2009). Activated microglia produce anti-inflammatory factors and can even sheathe neurons to provide trophic support under damaging or regenerating conditions. Chen et al. showed that microglial activation was not detectable 24 hours after the first LPS injection and microglial morphology was similar to that of the phosphate buffered saline injected animals. Twenty-four hours after the second LPS injection, microglia began to retract their processes and enlarge their cell bodies. Interestingly, numerous spiny protrusions were seen at this interval, giving them a "bushy" appearance, indicating transition to activation. One day after the fourth and final LPS treatment, microglia demonstrated asymmetrical process distributions, dense cell bodies and thickened proximal dendrites. With the increasing numbers of daily LPS injections, the microglial activation profile expanded throughout the cortical and subcortical gray matter. The density of the microglial cells was quantified by Iba-1 immunostaining.

Previous studies from *in vitro* and *in vivo* models demonstrated that acute alcohol

down-regulates LPS-induced production of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) and up-regulates anti-inflammatory cytokines, such as IL-10. (Boe et al., 2003) (Mandrekar et al., 1996). The released cytokines play a central role in antimicrobial defense; hence, the imbalance of pro- and anti-inflammatory cytokines after acute alcohol exposure contributes to increased susceptibility to infections (Goral and Kovacs, 2005), (Oak et al., 2006). Interestingly, Harris et al. had demonstrated that activation of immune signaling by LPS in C57Bl/6 mice also increases their prolonged alcohol consumption (Blednov et al., 2011). The authors report that LPS pretreatment produces long-lasting increase of ethanol intake in both male and female C57Bl/6 mice. C57Bl/6 male mice demonstrate an increase of ethanol intake even one month after LPS injection. CD14 is a co-receptor, along with Toll Like Receptor 4 (TLR4) for the detection of bacterial LPS. The authors also show that, CD14 null mutant mice do not show increased ethanol consumption after pretreatment with LPS. Thus, the mechanism of action of LPS to increase drinking appears to be mediated by TLR's as deletion of CD14 adaptor protein was sufficient to prevent an increase in ethanol consumption (Blednov et al., 2011).

Based on the above literature we proposed to characterize the role of NF-κB signaling in amplifying alcohol intake in a mouse model of LPS-enhanced alcohol consumption. We aimed to assess the importance of NF-κB activity in the macrophage compartment. Since, BV2 cells are the most frequently used substitutes for primary microglia we decided to use BV2 cells to perform our in vitro experiments.

4.2 Experimental Design and Methods

4.2.1 Reagents and cell culture

LPS from *Escherichia coli* (catalogue # L2630-100mg) was obtained from Sigma. All other reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. BV2 (a murine microglial cell line) cells was maintained in DMEM (Cellgro) medium supplemented with 10% fetal bovine serum (Atlas Biologicals, Inc.), 2 mM Glutamax (Life Technologies) and penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO_2.

4.2.2 SYBR green based quantitative PCR (Q-PCR)

Cells were plated at a density of 1X 10^6 cells/well in 6 well plates and allowed to adhere overnight. The cells were first stimulated with LPS (100 ng/ml) for 2 hour followed by treating with 100 mM ethanol in a time dependant manner. In another experiment, cells were stimulated with LPS (100 ng/ml) for different time points and treated with 100 mM ethanol for 1 hour. BV2 cells were also stimulated with LPS (100 ng/ml) and simultaneously treated with 100 mM ethanol for different time points. The cells were harvested and total mRNA was extracted using TRIzol reagent (Ambion) and RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) using the manufacturer's recommendations. Reverse transcription was performed using cDNA synthesis kit (Taqman, Life technologies). The RT-PCR analyses was performed using i taq™ Universal SYBR® supermix (Bio Rad) with 1 µl of cDNA, corresponding to 1 µg of total RNA in a 20 µl final volume, 8 µl H2O, 10 µl SYBR Green, 0.5 µl oligonucleotide primer pairs (synthesized at Sigma) at 1 mM (Table 2). PCR-program: 1. 25°C for 10 min, 2. 48°C for

30 min, 3. 95°C for 5 min, 4. 4°C forever. All samples were amplified in triplicates.

18S sense	5'-CGGGTGCTCTTAGCTGAGTGTCCCG-3'
18S antisense	5'-CTCGGGCCTGCTTTGAACAC-3'
TNF alpha sense	5'-TCTCATCAGTTCTATGGCCC-3'
TNF alpha antisense	5'-GGGAGTAGACAAGGTACAAC-3'
IL-6 sense	5'-TTCCATCCAGTTGCCTTCTTG-3'
IL-6 antisense	5'-TTGGGAGTGGTATCCTCTGTGA-3'
MCP-1 sense	5'-ATCCCAATGAGTAGGCTGGAGAGC-3'
MCP-1 antisense	5'-CAGAAGTGCTTGAGGTGGTTGTG-3'

Table 4.1: SYBR green primer pairs.

4.3 Results and Discussion

4.3.1 Analysis of the expression of genes after LPS and ethanol treatment

Alcohol consumption has been shown to increase in mice exposed to LPS but cells that participate in such behavior are still unknown. We hypothesized that LPS-induced NF-κB signaling in microglia was pivotal in potentiating the observed alcohol intake in the above described model. While we have not tested our hypothesis directly, we have begun to analyze this possibility by treating BV2 cells with LPS followed by

ethanol in three different ways. First, BV2 cells were stimulated with LPS (100 ng/ml) for a fixed time for 2 hours, the media was aspirated out and fresh media was added with 100 mM ethanol. The cells were incubated with ethanol for different time points. The cells were then harvested for RNA and analyzed by Q-PCR. We then treated another set of BV2 cells with LPS (100 ng/ml) for different time points. The LPS was aspirated out and fresh media was added. The cells were then treated with 100mM ethanol for 1 hour only. The cells were then harvested for RNA and analyzed by Q-PCR. Finally, we wanted to treat BV2 cells with LPS (100 ng/ml) and 100 mM ethanol simultaneously for different time points. The cells were harvested for RNA and analyzed by Q-PCR. Q-PCR was used to analyze the expression of pro-inflammatory cytokines like IL-6, TNF-α and MCP-1, as these genes are known to get upregulated upon LPS stimulation. BV2 cells stimulated with LPS and no ethanol treatment served as a control. However, we did not notice any significant difference in the expression of these genes (Figure 4.1) when treated with LPS for 2 hours and then ethanol.

Interestingly, we notice a dramatic decrease in the expressions of pro-inflammatory cytokines IL-6 and TNF-α at 2 hours. There was a decrease in the expression of MCP-1 genes too (Figure 4.2). When BV2 cells were treated with LPS and ethanol simultaneously we notice a remarkable decrease in the expression of IL-6 and TNF-α at 4 hours post stimulation (Figure 4.3). From our current study, we can conclude that upon BV2 cell stimulation by LPS followed by ethanol treatment there is a decrease in production of IL6, TNF-α. This suggests that ethanol is inhibitory to the production of inflammatory cytokines, suggesting the role of ethanol in regulating neuroinflammation.

4.3.2 Discussion

NF-κB has been long recognized as the master regulator of inflammation. The origins of many inflammatory-associated diseases, including cancer, have been liked to NF-κB (Grivennikov et al., 2010) and (Karin, 2009). Importantly, it has now been recognized that neuroinflammation is an important component in the etiology of alcohol-induced diseases. Alcohol consumption has been shown to increase in mice exposed to lipopolysaccharide (LPS), a bacterial cell wall component that binds and stimulates signaling of the Toll-like receptor-4 (TLR-4) pathway of which NF-κB is a downstream effector. Notably, mice null in components of the TLR4 signaling pathway have decreased alcohol-drinking behavior compared to control mice (Blednov et al., 2011). However, it is unclear as to which cell types of the brain contribute to the neuroinflammation that facilitates alcohol consumption and whether NF-κB is a major contributing factor in the observed neuroinflammation induced drinking model.

So, my goal was to assess the importance of NF-κB in contributing to the LPS drinking model. A mouse model has been proposed for enhanced alcohol consumption in this study. In this model, injection of LPS induces inflammation and promotes alcohol drinking. Since, the immortalized murine microglial cell line BV-2 has been used frequently as a substitute for primary microglia, in our initial studies, we stimulated the BV-2 cells with LPS in a time dependent manner and treated the cells with 100 mM ethanol for a fixed time. The cells were harvested for RNA and Q-PCR was done to monitor inflammatory gene expressions as readout for NF-κB activity. Interestingly, we have observed that ethanol is inhibitory to the production of inflammatory cytokines only

when cells were treated with LPS first for different time points and then treating with ethanol for an hour, thus suggesting the role of ethanol in suppressing neuroinflammation. Previously, ethanol has been shown to have inflammatory effects on the brain cells. However, Wang *et al*. has shown that ethanol at 100 mM suppressed LPS induced increase of nitrite levels in rat mixed glial cells (Wang et al., 1998). Ethanol selectively inhibited LPS-induced NF-κB activation, but not IFNγ-induced signaling (Lee et al., 2004). Now our current results in BV2 cells also support this view, that ethanol at a dose of 100 mM is inhibitory to the production of inflammatory cytokines.

In the recent future we plan to establish an *in vivo* inflammatory model in mice to test our hypothesis that, depending on the combination and the order the BV2 cells receive LPS/Ethanol, they will secrete specific cytokine signatures, which will affect neurons in a specific way. This project is of importance because understanding the molecular underpinnings of inflammation-induced drinking will provide a foundation for future strategies to block neuroinflammation and possibly curb drinking behavior.

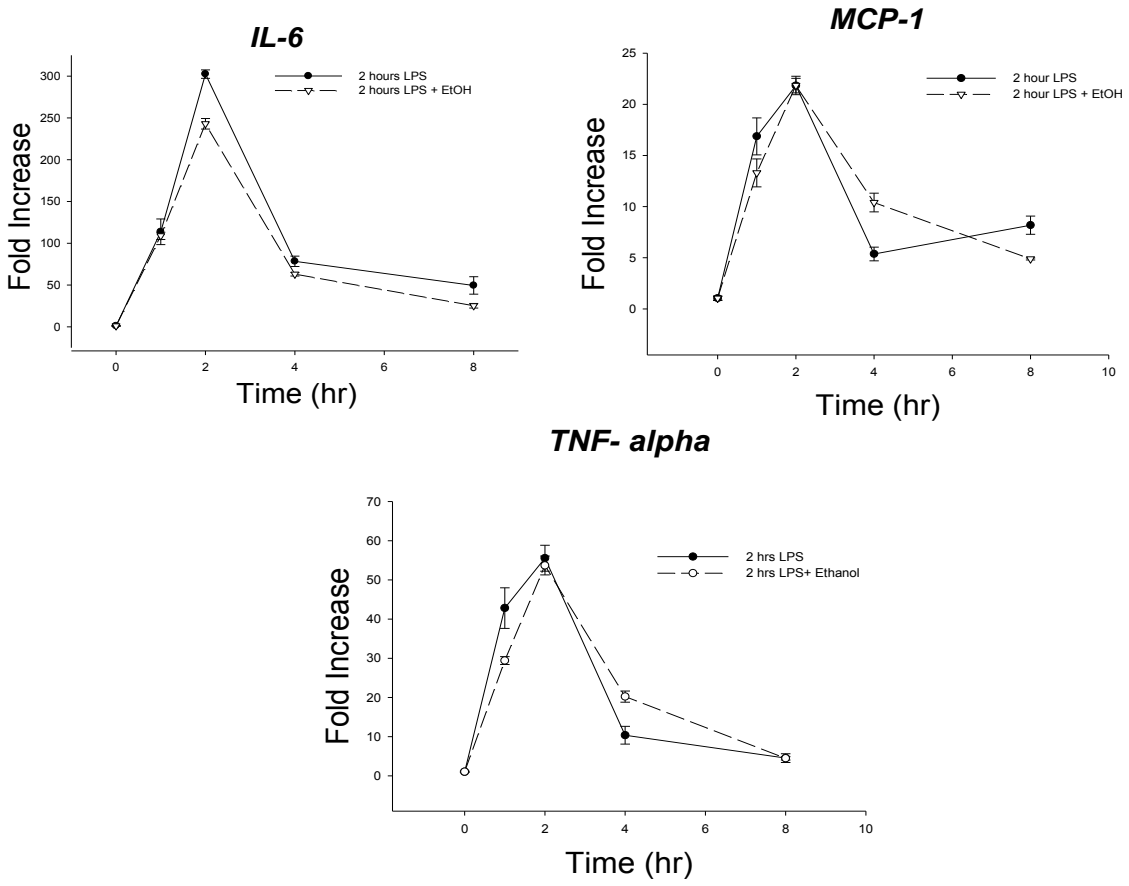


Figure 4.1: Q-PCR to analyze the expression of inflammatory genes after a fixed 2 hours LPS treatment followed by ethanol treatment for different time points.

BV2 cells were stimulated with LPS (100 ng/ml) for a fixed time for 2 hours, the media was aspirated out and fresh media was added with 100 mM ethanol. The cells were incubated with ethanol for different time points. The cells were then harvested for RNA and analyzed by Q-PCR.

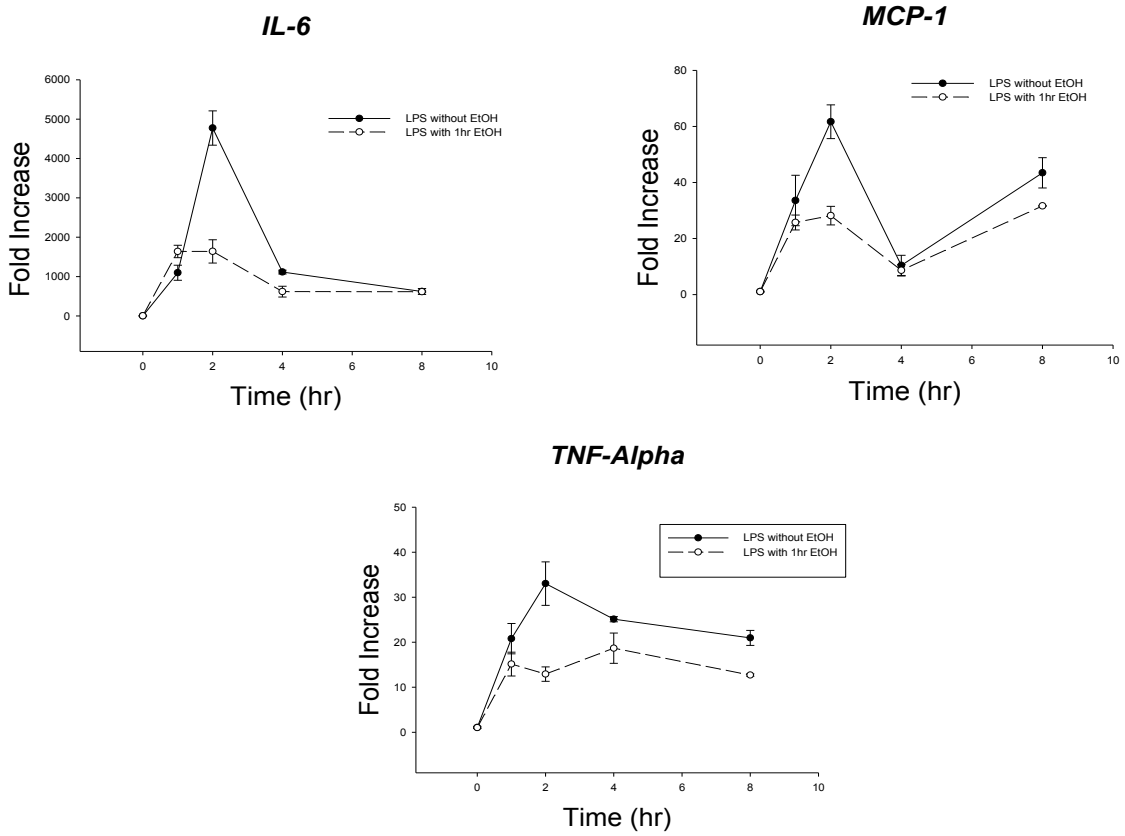


Figure 4.2: Q-PCR to analyze the expression of inflammatory genes after LPS treatment for different time points followed by ethanol treatment for 1 hour.

BV2 cells were stimulated with LPS (100 ng/ml) for different time points. The LPS was aspirated out and fresh media was added. The cells were then treated with 100mM ethanol for 1 hour only. The cells were then harvested for RNA and analyzed by Q-PCR.

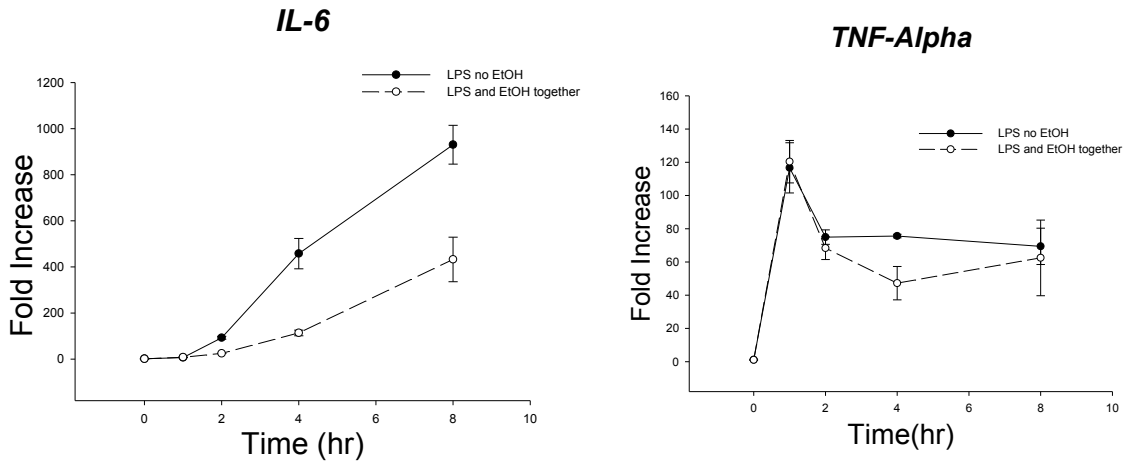


Figure 4.3: Q-PCR to analyze the expression of inflammatory genes after treating with LPS and ethanol simultaneously.

BV2 cells were stimulated with LPS (100 ng/ml) and simultaneously treated with 100 mM ethanol for different time points. The cells were harvested for RNA and analyzed by Q-PCR.

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