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Characterization of B-Lymphoblast Lines Derived from a Severely Autistic Child, Both Parents, and Donor Controls

Delia Juliana
Clemson University, deliaj@g.clemson.edu

CHARACTERIZATION OF B-LYMPHOBLAST LINES DERIVED FROM A SEVERELY AUTISTIC CHILD, BOTH PARENTS, AND DONOR CONTROLS

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Delia Juliana
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Dr. Charles Rice, Committee Chair
Dr. Lisa Bain
Dr. Yanzhang Wei

ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition affecting approximately 1% of the population. It occurs roughly four times more in males than females, and includes individuals with classic autism, as well as those with “incomplete” forms. Behavioral characteristics include, but are not limited to: restricted, stereotyped, and repetitive actions, as well as impairments in communication and social interaction. At the Greenwood Genetics Center, Greenwood, SC, ASD patients and control donors provided peripheral blood as a source of lymphocytes, and a skin-scrape sample as a source of fibroblasts for genetic analysis. A severely autistic patient, coded as B26, his two parents (mother Y17, father VT8), and two donor controls (JOR, RT9) were compared. Patient and donor lymphocytes were transformed into immortal lymphoblast lines using EBV-transformation procedures. In addition to routine genetic analysis, both fibroblasts and lymphoblasts were evaluated by GGC researchers as a possible platform to generate neuronal cells through iPS steps. Attempts were successful with fibroblasts, but not lymphoblasts. In this thesis, these five lymphoblast lines were evaluated for possible association with ASD and both parents, along with two donor controls. These cells were also examined for their potential in other areas of research, including the autocrine production of inflammatory and anti-inflammatory cytokines, reactivation of RAG-1 as a consequence of EBV-transformation, pharmacological and toxicological applications mediated through the AHR, and their metabolic responses to

tryptophan exposure. All five cell lines produce significant quantities of the autocrine B-cell growth factor IL-6, and, to a lesser degree, IL-10, before and after further activation with phorbol ester in combination with calcium ionophore. Patient B26 and his two parents produced the most cytokine in response to activation, while the control donors had a higher baseline production. All five cells lines expressed RAG-1 protein, indicating reactivation of B-cell receptor diversity process that may lead to a different BCR specificity. In pharmacology and toxicology, many xenobiotics and endogenous metabolites of tryptophan act through the AHR leading to induction of phase I, II, and III enzymes. The classical marker of AHR activity is the induction of CYP1B1. In response to FICZ, a potent tryptophan intermediate, patient B26 had the highest level of CYP1B1 induction, followed by Y17, the mother. Because tryptophan is metabolized by IDO2 in activated normal lymphocytes, the induction of IDO2 was quantified after activation with phorbol ester and calcium ionophore. Expression of IDO2 was highest in patent B26, followed by Y17, the mother. Recent research at GGC suggests that tryptophan is metabolized differently in ASD patients when compared to donor controls. A commercially available PCR array of genes associated with tryptophan metabolism was then employed and revealed that key genes involved in tryptophan metabolism are not altered in the severely autistic patient B26, either of B26's two parents (mother Y17, father VT8), nor the two donor controls (JOR, RT9). The array also showed that several genes involved in mitochondrial function were highly responsive to tryptophan

treatment in B26, and similarly in the mother, Y17. Genes not only involved in energy metabolism, but also inflammation and different pathways stemming from tryptophan metabolism (serotonin and melatonin pathways) were increased. This study shows that lymphoblasts generated from ASD patients and donor controls may have use outside of the field of autism research, and may lead to the development of novel biomarkers for screening individuals for ASD.

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LITERATURE REVIEW

ASD is a behavioral syndrome that is usually revealed during early childhood and is hypothesized to reflect underlying neurodevelopmental abnormalities [1]. Core symptoms of ASD include abnormal or unreciprocated interpersonal interactions, muddled language and communication, and repetitive and stereotypic behavior [2]. Several biomarkers have been associated with ASD, but there are currently no laboratory tests to be used as concrete diagnostic tools [3]. The etiology of ASD has brought upon much divisiveness among scientists and the general public in recent years. There has been a dramatic climb in the number of children diagnosed with one of the ASDs, with the most severe form being autistic disorder [4]. In the United States alone, the diagnosis of ASD has increased almost ten-fold over the last decade [5]. This increase is seen as alarming because in the 1980's, there were roughly 4-5 children per 10,000 diagnosed. In the 1990's, there were about 30-60 children diagnosed per 10,000 people [6]. Some scientists believe that a main cause of this upsurge is the result of heightened awareness of ASD now compared to the 1980s. Some believe that it is the change in diagnostic criteria, suggesting that the number of people with ASD has stayed stable over time [7]. There are few studies that have been designed to definitively address whether the true prevalence of ASD has stayed stable or increased during time, but many agree that it would be premature to state that there is no increase in prevalence [8]. Most diagnoses are based upon behavioral symptoms, which have been well

described. Much less research is focused on the causes of these symptoms. This begs the question of why - why are the core symptoms of ASD social disinterest, problems in communication, and overly focused behavior? Where do these behaviors internally stem from? The goal of this literature review is to highlight the relevance of prior studies in the development of this thesis as it pertains to ASD from a genetic standpoint, as well as cytokine involvement and AhR activation in ASD patients.

A previous study analyzed the metabolic profile of lymphoblast cell lines from 137 patients with neurodevelopmental disorders with and without ASD and 78 control individuals [9]. This and another study looked at individuals with ASD versus control patients, and saw that the ASD patients exhibited on average reduced generation of NADH when tryptophan was the sole energy source [10]. The results correlated with the behavioral traits associated with either syndromal or non-syndromal autism, but it did not seem to matter what the genetic background of the individual was [11]. A low level of NADH generation in the presence of tryptophan was observed in cell lines from ASD patients, but not in cell lines from non-ASD patients with either intellectual disability, schizophrenia or similar conditions to ASD except for the behavioral traits [12].

Analysis of a previous small gene expression study found abnormal levels for some genes involved in tryptophan metabolic pathways in 10 patients [13]. It can be hypothesized that the observed reduction of tryptophan metabolism in ASD cell lines may come from any abnormal function along tryptophan metabolic

pathways in the cells [14]. Some of the most frequently reported findings in ASD brains are increased brain size and increased white matter [15]. It is possible that impairment of the kynurenic pathway may have consequences on the pruning process in the brain and lead to an excess of white matter [16]. Microglial cells are considered to be responsible for keeping the proper balance between quinolinic and kynurenic acid, since they are able to secrete both compounds [17]. The expression of those molecules is strongly influenced by the activity of the immune system, which has been implicated as impaired in ASD individuals in a number of studies.

Tryptophan is also the main precursor for both melatonin and serotonin. Melatonin plays a critical role in the regulation of the circadian rhythm, and disturbances in this rhythm have been associated with several symptoms in ASD, like seizures or sleep disorders [18]. Serotonin is an important neurotransmitter involved in many functions, from the regulation of mood to the control of appetite and social interactions, and its production has been reported as deficient in the brains of ASD individuals [19]. Since tryptophan levels have been shown to influence central nervous system serotonin levels and behavior, it can be concluded from these studies that an altered tryptophan transport exists in patients with ASD [20].

The cell lines studied included a severely autistic child, B26, as well as the two parents of the patient with ASD and two donor controls (JOR and RT9). B26 has inherited microdeletions on chromosomes 10p12.31 and 15q15.3 from each

parent respectively. These microdeletions on the corresponding chromosomes have been associated with developmental delay, low muscle tone, and communication difficulties, and ASD. It would be advantageous to know how, or if, a combination of chromosomal deletions could be linked to the severity of ASD, and to look at the evolution of genome sequencing. In the twentieth century, knowledge about human genetic variation was limited mainly to the heterochromatin polymorphisms, and single nucleotide polymorphisms (SNPs) identified by traditional PCR-based DNA sequencing. In the past ten years, the swift development and extended use of microarray technologies, such as the commercial array used in this study as well as next-generation sequencing, has enabled a whole-genome analysis with essentially unlimited resolve [21]. The discovery of submicroscopic copy-number variations (CNVs) present in our genomes has changed the perspective on DNA structural variation and disease. CNVs have been shown to be responsible for susceptibility to traits (genomic disorders), genetic diversity, and human evolution. In addition, many common complex traits including autism can result from CNVs. Since CNVs can involve part of a gene or even several genes, some CNVs are likely to have a role in the alteration of human physiological functions, causing disease [22]. They therefore are likely to be subject to evolutionary pressures such as selection and genetic drift.

Altered immune response in ASD individuals has been reported for almost 40 years [23], and there is evidence that suggests increased inflammation in the

brain and CNS based upon specimens obtained from individuals with ASD [24]. Several studies have shown immune differences in children with ASD, such as abnormal cytokine profiles [25]. A subset of studies have demonstrated increased levels of inflammatory cytokines in ASD, such as IL-6 and increased levels of IL-10 [26]. However, varied experimental designs, diagnostic criteria, varying ages in comparison of ASD patients with donor controls (children with ASD vs. adult controls) have caused misinterpretation of the results [27]. Small sample sizes have posed a challenge in the ability to detect differences in cytokine patterns between ASD patients and donor controls, and this is showcased in the current study.

Lastly, countless studies have indicated that cells from patients with ASD, on average, are less capable of utilizing tryptophan as an energy source than controls. Although not published yet, a study by Dr. William Baldwin has showcased that B26 fibroblasts contain more mitochondria but are insufficient at mitochondrial repair and response to oxidative stress. This is shown through an array of important genes in tryptophan metabolism. Moreover, findings in these studies were consistent in patients across the autism spectrum and was not influenced by age or sex. Decreased tryptophan metabolism in patients with ASDs may alter metabolic pathways involved in the regulation of the early stages of brain development, mitochondrial homeostasis and immune system activity in the brain [28].

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition affecting approximately 1% of the population. It occurs roughly four times more likely in males than females, and includes individuals with classic autism, as well as those with “incomplete” forms of autism [29]. People with ASD fall on a spectrum between low functioning and high functioning, and the severity ranges from patient to patient. Behavioral characteristics include, but are not limited to: restricted, stereotyped, and repetitive actions, as well as impairments in communication and social interaction. A strong genetic predisposition for ASDs has been inferred from a higher concordance in identical twins (between 30%-60%), as compared to fraternal twins (roughly 6%) [30]. The higher concordance rate found in identical twins suggests that multiple genes are related to the cause of autism. The combination of affected genes and the environment both play a role in the development of ASD. There is no particular laboratory test to diagnose ASD, although several biomarkers have been associated with it [31]. The cause of ASD is not known and likely involves interactions between environmental, and most importantly, genetic factors.

Links to environmental factors in the development of ASD focus on contaminants involved in air pollution, and especially persistent organic pollutants (POPs). This includes PCBs, dioxins, and furans, as well as some polycyclic aromatic hydrocarbons, such as benzo-*a*-pyrene, which is also found in secondary smoke from tobacco use [32]. Many of these environmental

pollutants are ligands for the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor that leads to the induction of phase I xenobiotic metabolizing enzymes, such as CYP1B1 and CYP1A1 [33]. While no studies have linked the expression of, or genetic variants of AHR with causes of autism, and no studies have shown differences between AHR expression and variants in control vs. patients, there is a correlation between expression and severity score [34]. One of the most potent of AHR ligands is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and exposure is associated with a plethora of syndromes, including wasting, hepatocellular carcinoma, chloracne, reproductive toxicity, thymic atrophy, and immune dysfunction; the underlying mechanism may be the loss of NAD+ [35]. In some ASD patients, altered tryptophan metabolism also leads to the loss of NAD+ [36], which may hint to altered AHR signaling in some ASD patients. Moreover, the induction of CYP1B1 and CYP1A1 by some environmental pollutants may lead to extremely toxic, mutagenic, and teratogenic intermediate metabolites. To date, little is known about the role of AHR, phase I enzyme activities, and ASD.

The expression of CYP1B1 can be induced by a number of endogenous ligands, including tryptophan and its metabolites through the AHR [37] [38]. In addition, CYP1B1 is involved in the metabolism of many important physiological compounds including arachidonic acid, estrogen, melatonin and retinoids [39]. In previous research, genes that are known to be involved in tryptophan metabolism, such as CYP1B1, were examined in a small subset of patients with

ASD. It was found that these patients expressed CYP1B1 at higher levels than those without autism. A previous study done by Smerdova et al. demonstrated that there was an upregulation of CYP1B1 expression in ASD individuals because of an increase in inflammatory cytokines and aryl hydrocarbon ligands [40].

Recent papers have described links between genes that encode for immune-related proteins such as IL-6 and IL- 10 and ASD, suggesting that abnormalities in the immune system may impair brain development [41]. Interleukin-6 was originally labeled B-cell growth factor because it is critical to the growth and proliferation of myeloma cells, but is also known as a classical marker of macrophage-associated inflammation, and known to be required for normal neurological development and physiology [42, 43]. Intereukin-6 is of interest because studies have suggested that brain IL-6 is an important mediator of autism-like behaviors [44] [45].

Current evidence dictates that IL-6 may have a crucial role within the CNS. In the CNS, IL-6 can trigger cellular responses such as inflammation, cell growth, neurogenesis, and cell survival among other processes [46]. IL-6 is ordinarily expressed at reasonably low levels in the brain, but in the presence of injury or inflammation, IL-6 is elevated in spinal fluid as well as brain homogenate. In a study done by Wei, H. et al., it was shown that chronic overexpression of IL-6 in mice causes anatomical and physiological changes in the brain, leading to disorders such as ASD [47].

Interleukin-10 (IL-10) is a well-known anti-inflammatory cytokine that plays an important role in preventing inflammatory pathologies [48]. Gerber et al. found that an increase in cathepsin D, a protease abundantly expressed in the brain, can trigger secretion of cytokines, including IL-10 due to a corresponding increase in pro-inflammatory cytokines, such as IL-6 [49]. This could suggest that IL-10 acts in a negative feedback loop, or a homeostasis, for when levels of IL-6 increase.

Some research suggests that patients along the ASD scale have an altered ability to utilize tryptophan [50]. Tryptophan is an essential amino acid and needs to be supplemented via diet. It is also an AhR agonist and precursor of many important compounds such as kynurenic acid, serotonin, and quinolinic acid – all of which are involved in neurodevelopment and the formation of synapses [51].

The majority of tryptophan is metabolized along the kynurenine-quinolinic pathway of tryptophan degradation, and follows two other major pathways of metabolism in the GI tract: transformation by microbiota in the gut of tryptophan into ligands of the aryl hydrocarbon receptor (AhR), and the serotonin production pathway [52]. This is demonstrated in illustration 1. Decreased tryptophan production seen in ASD individuals may alter development of the brain, immune activity, and mitochondrial function. Studies have shown that the abnormal utilization of tryptophan as an energy source further supports possible mitochondrial dysfunction [53].

Metabolism of tryptophan via the kynurenine-quinolinic pathway leads to the synthesis of NAD+, while the serotonin branch of the tryptophan metabolic pathway generates NADH. Serotonin functions as a key neurotransmitter and serves as a communication system between the central nervous system and the GI tract [54]. The microbial impact on the metabolism of tryptophan and the serotonin pathway may be important in the regulation of the brain-gut axis. It has been previously concluded that individuals with ASD may have a decreased diversity of gut microbiota, dictating serotonin-related health problems [55]. This could be related to the ability of the microbiota in the gut to control metabolism of tryptophan along the kynurenine pathway, therefore reducing the amount of tryptophan needed for serotonin synthesis.

Observing decreased levels of NADH generation in the presence of tryptophan may indicate reduced utilization of tryptophan resulting from downregulation of metabolic reactions at any point along the two pathways. The kynurenines produced during the kynurenine-quinolinic pathway via indoleamine 2,3-dioxygenase 2 are potent ligands for the AhR, and are involved mainly in inflammatory response, immune response, and excitatory neurotransmission [56]. Indoleaminedioxygenases 1 & II (IDO1, IDO2) are induced by various pro-inflammatory cytokines (such as IL-6). Therefore, in immune-activated environments, IDO may break down a large proportion of tryptophan leading to a shortage for the serotonin–melatonin pathway. It has been observed that in some ASD patients, there is a dysfunction in a number of genes involved in the

kynurenine-quinolinic pathway and the serotonin pathway compared to donor controls, namely the production of IL-6 [57].

As previously mentioned, a portion of tryptophan is metabolized in the GI tract and transformed by microbiota in the gut into ligands of the AHR. Since the AHR contains a promiscuous ligand-binding pocket, it can bind multiple classes of exogenous compounds. The photo-oxidation product of tryptophan, 6-formylindolo(3,2-b) carbazole, otherwise known as FICZ, is one of the more potent endogenous ligands for the AHR and may be the primary metabolite of indole-3-carbinol (I3C) during digestion, which has been found to modulate the expression and activity of biotransformation enzymes involved in metabolism and excretion of many biologically active compounds [58]. In addition, FICZ induces AHR translocation to the nucleus and transcription of the AHR target gene CYP1B1, as demonstrated in illustration 2. Polymorphisms of some AHR-induced genes in ASD individuals may cause an increase in CYP1B1 production, therefore a possible increase in severity of ASD [59]. The expression of indoleamine dioxygenases (IDO) is involved in catabolism of tryptophan and is believed to be a major source of tryptophan dysfunction in ASD patients. In other studies, it is hypothesized that there is an increased level of IDO, resulting in an increased catabolism of tryptophan [60]. This means that tryptophan is possibly metabolized too quickly and being delegated to the AHR rather than other tryptophan pathways such as the serotonin pathway.

While progress in ASD research has been made, where studies have attempted to evaluate whether cytokine levels are associated with onset of stereotypical behaviors in ASD, there are still many shortcomings in finding a definitive diagnostic tool in a clinical setting for a concrete diagnosis. As of now, there are no laboratory tests that can accurately diagnose ASD. Therefore, diagnosis is based upon observed stereotypical behaviors associated with ASD. Many children are not diagnosed until the age of 2 or 3, meaning that the window to intervene early is essentially closed before a diagnosis is even made [61].

Researchers at the Greenwood Genetics Center, Greenwood SC USA, are intensely focused on autism research. As part of a larger, long term study, ASD patients and control donors have provided peripheral blood as a source of lymphocytes, and a skin-scrape sample as a source of fibroblasts, both for genetic analysis. Patient and donor lymphocytes were transformed into immortal lymphoblast lines using EBV-transformation procedures. In addition to routine genetic analysis, both fibroblasts and lymphoblasts were evaluated by GGC researchers as a possible platform to generate neuronal cells through iPS steps. Attempts were successful with fibroblasts, but not lymphoblasts. However, these lymphoblasts lines from ASD patients and donor controls may hold promise in aspects of autism research outside of iPS-derived neuronal cells, or even outside of autism research.

EBV-transformed B-cells to lymphoblasts are known to secrete IL-6 in an autocrine fashion to stimulate continuous proliferation [62], and levels can be

increased by further activation with phorbol esters (e.g., phorbol ester myristate – PMA). It is now known that activiation can be improved by the addition of calcium ionophore (e.g., A23187, ionomycin), and this combination is routinely used in current research protocols. The addition of PMA activates Calcium/phospholipid-dependent PKC, and calcium ionophore A23187 increases intracytosolic free calcium concentration [63]. Together, PMA and A23187 bypass the early activation pathways and promote B-cell growth through the direct induction of membrane IL-6 receptor expression and IL-6 synthesis and secretion. Also, EBV-lymphoblast-derived IL-6 has been used as a source to stimulate the growth of human myeloma cells. These cells also secrete IL-10 as a negative control against over stimulation by IL-6 [64]. It has been observed that in some, but not all EBV-transformed lymphoblast lines, that RAG-1/RAG-2 genes are reactivated in terminally activated lines from continuous culture, and may be an indication of full activation [65].

Little is known about the AHR activities in EBV-derived lymphoblasts, including baseline AHR expression levels and induction of CYP1B1 by AHR ligands, but one study showed that AHR activation leads to EBV-reactivation in latent infected B-cells, which may lead to Sjogren's Syndrome [66]. The questions of how do EBV-transformed lymphoblasts respond to AHR ligands is an open question, and fertile ground for future research. Moreover, this is even more so for EBV-transformed lymphoblasts derived from ASD patients.

To my knowledge there is no consensus on how tryptophan is metabolized by EBV-transformed B-cells in general, but metabolism of this critical amino acid during other chronic viral diseases has been examined [67]. Tryptophan depletion by IDO and AHR both depletes tryptophan needed by the pathogen, and contributes to the state of local immunosuppression induced by metabolites, because prolonged activation of the AHR leads to immunosuppression [67]. Whether or not tryptophan metabolism in chronically activated EBV-transformed lymphoblasts from a severe ASD patient is different than donor controls is one of the key questions asked in my thesis.

The purpose of the research for this thesis is to characterize lymphoblasts cell lines derived from ASD patients and control donors to determine if there are intrinsic differences in levels of secretion of autocrine growth factors IL-6 and IL-10, in states if activation, expression of AHR and CYP1B1 involved in the metabolism of endogenous indoles, and differences in gene expression pathways involved in tryptophan metabolism. A severely autistic patient, coded as B26, his two parents (mother Y17, father VT8), and two donor controls (JOR, RT9) were compared. It is my hope that this information will be a spring board for the next set of approaches using these cell lines, and for the development of possible screening biomarkers for differences between ASD patients and those not diagnosed with ASD.

METHODS

Study subjects

First, peripheral blood lymphocytes from ASD patients and donor controls were provided to Greenwood Genetics Center and transformed to immortal B-cell lymphoid cell lines using EBV transformation. Cells from each subject were coded by internal referencing numbers, and later given an abbreviated name. This study focuses on one patient with severe ASD having deletions 10p12.31 and 15q15.3, inherited from the mother and father, respectively. The patient with ASD was given the code B26. The patient's parents were given code Y17 (mother) and VT8 (father), and the other two donor controls were coded RT9 and JOR.

The following general procedure for transforming B-cells to lymphoblast is as follows. First, mL of blood was drawn from each donor and diluted with 20 mL of PBS at room temperature. The diluted blood was subsequently put into separation media and centrifuged at 225 x *g* at room temperature for 30 minutes. Concentrated white blood cells that collected at the top were removed, and the sample was diluted with PBS up to 50 mL. Samples were spun down once more at 600 x *g* at room temperature for 10 minutes. After centrifugation, supernatant was poured off, and the cells were washed by re-suspending the pellet in 50 mL PBS, and spinning down at the same settings. This was repeated twice more. Once the cells were washed, they were re-suspended in 1 mL of fresh media. Ten microliters of cells were used for counting.

Infection with EBV

Methods for transforming B-cells followed previously published methods [68, 69], and carried out by researchers at the Greenwood Genetics Center (GGC), Greenwood, SC USA. Exponentially growing B95-8 cells (ATCC # CRL 1612; 13) at 3 x 10^5 cells/ml in a 75cm^2 tissue culture flask were maintained using sterile technique. Cells are grown in complete RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), Penicillin/Streptomycin at 100U/ml and 100 µg/ml, respectively, and Amphotericin B at 0.5 µg/ml at 37°C in the presence of 5% CO_2. To ensure virus production, the cells were stimulated with 20ng/mL tetradecanoyl phorbol acetate (TPA; a.k.a. PMA) for 1 hour in a standard CO_2 incubator. The cells were then washed three times with the media to remove the TPA. Cells were re-suspended in the same amount of fresh media previously mentioned and placed in a flask inside of the incubator for 96 hours. After the incubation time, cells were centrifuged at 600 x *g* in order to separate the supernatant containing EBV. The supernatant containing virus was then filtered out through a 0.45 µn filter, partitioned, and stored at -70°C. Peripheral blood cells from patients and donors were mixed with virus-containing supernatants as previously described, and stimulated with phyohemmaglutin-A (PHA). FK506, a T cell immunosuppressant, was added to the cell suspension from above, and placed into the incubator at 37°C for 1 hour. After incubation, an aliquot of EBV previously prepared was added to the flask at a 1/10 dilution. The flask was swirled and sat upright in the incubator for 3 days. Cells were

subsequently cloned and verified to be B-cell blast using flow cytometry with B-cell-specific antibody markers.

Both the ASD and donor control human lymphocyte cell lines were stored at GGC, and also maintained at Clemson University. Cells are grown in Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 20% heat inactivated fetal calf serum (Thermo Fisher), 25mM HEPES, 4.5 g/L glucose, 110 µg/mL sodium pyruvate, 10 mM L-glutamate, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mg/mL Gentamicin, 10 mg/mL Nystatin, 1% MEM Non-Essential Amino Acids (100X), and 7.5% sodium bicarbonate, each from Sigma (Sigma Aldrich, St. Louis MO, USA). Cells were grown and maintained at 37°C in a standard incubator in 75 cm^2 culture flasks.

Expression of IL-6 and IL-10 in untreated and stimulated cell lines

Cell lines were maintained prior to manipulation as described above. Two separate conditions were investigated. In the first set or experiments, cells were seeded at 2 x 10^6 cells/mL in T-25 cm^2 Corning culture flasks, in triplicate, and supernatants collected at the start of culture, and again at 6 and 12 hr to determine baseline production of IL-6 and IL-10. This was to determine the relative rate of production by each line, but also to determine the relative magnitude of production. In the second set of experiments, the same culture conditions were maintained, except that each cell line was treated with either 0.01% DMSO containing media, or the same with the addition of 1 uM PMA + 0.5

M A23187, which bypasses the B-cell receptor signaling pathways to activate PKC-delta, NFK-beta, and AP-1 to stimulate production of cytokines. IL-6 and IL-10 were both quantified by Human IL-6 ELISA MAX Deluxe Set (Biolegend) and Human IL-10 ELISA MAX Deluxe Set (Biolegend) respectively, following directions provided by the supplier.

Expression of RAG-1 and AHR protein

Two million lymphoblasts were seeded in T-25 flasks in 3 ml of media and incubated for 24 hr. Cells were removed from each flask and pelleted by centrifugation and covered with 500 µl RIPA lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Pierce). The cell pellet was disrupted by gentle vortexing, and incubated on ice for 30 min. Thirty micrograms of whole cell lysate protein from each sample were separated by SDS-PAGE on 4-20% gels (Biorad) and transferred overnight onto 0.2 PDVF membranes (Thermofisher) at 4 °C. For RAG-1 detection, whole mouse thymus lysate (Novus Biologicals) was examined as a positive control. For AHR detection, cell lysates from the human glioblastoma cell line T98G (ATCC) were used due to the high amount of AHR expressed in these cells [70]. The membranes were washed three times with 0.01 M phosphate buffered saline (PBS) containing 0.1% tween-20 (PBS-tw20), covered with blocking buffer (10% FCS in PBS) for 1 h and again washed three times with PBS-tw20. Membranes were then probed for 2 h with either rabbit

anti-human RAG-1 antibody (RNF4; Abcam) at 2 ug/ml, mouse monoclonal anti-humann AHR (mAb A-3; Santa Cruz) at 2 ug/ml, or mouse monoclonal anti-β actin (Sigma Aldrich) at 2 ug/ml. Antibodies were diluted in PBS-tw20 containing 1% FCS. The blots were washed three times for 10 min with PBS-tw20, and probed for 2 h with either goat anti-rabbit IgG, or goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (ThermoFisher) diluted 1:1000 in PBS-tw20 containing 1% FCS. After extensive washings with PBS-tw20, alkaline phosphatase activity was determined using the substrate NBT-BCIP to visualize the relative amount of specific protein.

Expression of CYP1B1 and IDO2 mRNA

To quantify CYP1B1 and IDO2 mRNA expression, each cell line was cultured in T-24 cm^3 flasks as described above, and treated with 10 nM FICZ (for CYP1B1 expression), or 1 uM PMA + 0.5 M A23187, (for IDO2 expression) for 12 hr. PMA + A23187 by passes the B-cell receptor signaling pathways to activate PKC-delta, NFK-beta, and AP-1 to stimulate IDO2 expression. The treatments were done in triplicate for each cell line. Following the treatment regimen, each sample was centrifuged, and a pellet was formed from each of the three replicated experiments. RNAzol (Molecular Research) was added (1 mL) to cell pellets, and the mixture was homogenized by pipetting gently several times. Samples were stored at -4°C until ready to use. Total RNA was isolated using protocol supplied by the manufacturer. First strand cDNA synthesis was carried

out using the RT² Easy First Strand Kit (SABioscience Corporation) as outlined by the manufacturer. Gene expression was evaluated by quantitative PCR with a BioRad iC5 detection system, RT² SYBR green master mix, and primer sets designed using Integrated DNA technology software. The primer sets were validated prior to use, and the quantity of mRNAs was expressed as fold changes using GAPDH as a housekeeping gene. Primer sets and relative information are as below.

Gene name	Accession #	Primer sequence (5' è 3')	Tm (°C)	Product size (bp)
IDO2	NM_194294	F:TGGAAATTGGGAACCTGGAGACCA R:ATCCCAGGCACTGCTTTCTCT	57	106
Cyp1b1	NM_000104	F: ATG TCC TGG CCT TCC TTT ATG R: GTG TCC TTG GGA ATG TGG TAG	52	115
Gapdh	NM_001256799	F: AGC CTC AAG ATC ATC AGC AAT GCC R: TGT GGT CAT GAG TCC TTC CAC GAT	57	105

Table 1. Primer sets for quantifying CYP1B1 and IDO2 induction

For data analysis, the ΔΔCt method was used, and data was compared between treatments using ANOVA.

Commercial qPCR array for genes involved in tryptophan metabolism

Lymphoblastoid cells were seeded at 2 x 10⁶ cells/mL in 75 cm² Corning culture flasks. Cells were then treated for 24 h with 2 mM tryptophan as recommended by others [71]. This experimental regimen was repeated three times and the lymphocytes were pooled (n = 3) per treatment regimen, then collected after centrifugation to have one pooled pellet from each of the three

replicated experiments. One milliliter of TRI-reagent® (Molecular Research) was added to cell pellets and the mixture was homogenized by gently pipetting several times. The homogenate was incubated at room temperature for 5 min and 0.2 mL chloroform was added. Total RNA was then isolated via manufacturer instruction. After collecting RNA from each tube, DNA contamination was removed using an elimination mixture supplied by the manufacturer, and first strand cDNA synthesis was carried out using the RT2 Easy First Strand Kit (SABioscience Corporation) as described by the manufacturer. A predesigned Amino Acid Metabolism ProfilerTM array of 96 genes was purchased from SuperArray Bioscience Corporation for use with RT2 Real-Timer SYBR Green/ fluorescein qPCR master mixes purchased from the same supplier. Specific methods followed those suggested by the manufacturer. QPCR was performed on a BioRad iQ5 real-time PCR detection system. For data analysis, the ΔΔCt method was used; for each gene, fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. Data were gathered and interpreted using software provided by SABioscience. The goal was to determine which genes may be of interest to follow up with, confirmed using regular qPCR.

RESULTS

IL-6 and IL-10 expression in untreated cell lines, and cell stimulated with PMA + A23187

Interleukin-6 secretion in lymphoblast lines was compared to background levels in complete media, and shown vary from line to line, but was higher in both control donors than in B26 and the two parents (Figure 1A). This was the case for both 6 hr and 12 hr incubation. Under these conditions donor JOR produced the most IL-6, followed by donor RT9. Interleukin-10 secretion by lymphoblasts under the same culturing conditions was higher than IL-6, with the two donor controls secreting the most (Figure 1B). At 6r, IL-10 secretion by VT8 (the father) was also high, and nearly matched the two donor lines. By 12 hr, IL-10 levels in the father's line (VT8) were lower, as were the others.

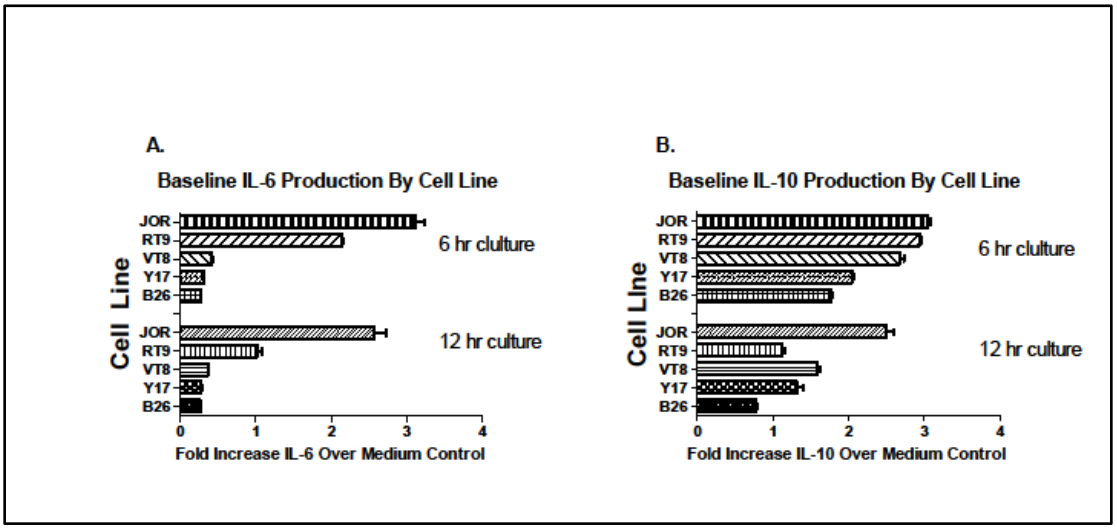

Figure 1. Interleuking-6 and IL-10 secretion by lymphoblast cell lines at 6 hr and 12 hr culture. Cells were grown in complete media only, and without further

treatment. Cytokine levels were quantified by ELISA, and the data represent the mean +/- S.E. of levels above that measured in complete media without cells.

In response to PMA + A23187, IL-6 and IL-10 secretion was quantified as an indicator of the ability of lymphoblast to increase production beyond background. Cells generated from patient B26 responded with a robust increase in secretion of IL-6 secretion at 12 (Figure 2A), with the expected increase over time. Likewise, Y17 cells (the mother) responded to stimulation with a large increase in production over time (Figure 2B). This was also observed in VT8 cells (the father) in response to stimulation over time (Figure 2C). Cells derived from the two donor controls did not respond as well as the other three, with RT9 producing a modest amount at 6 hr, and much less at 12 hr (Figure 2D), while donor JOR produced very little IL-6 at 6r, and nothing significant at 12 hr (Figure 2E). These observations are nearly reversed of what was produced by unstimulated cells (Figure 2).

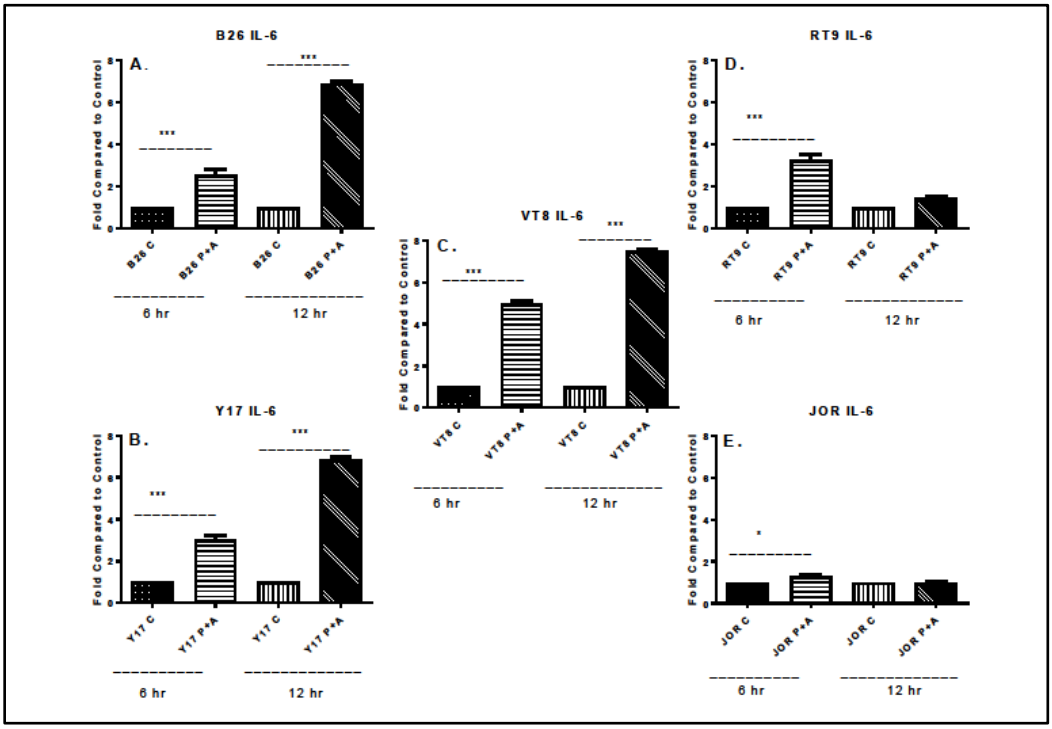

Figure 2. Interleuking-6 secretion by lymphoblast cell lines at 6 hr and 12 hr culture after stimulation with PMA + A23187. Cytokine levels were quantified by ELISA, and the data represent levels above non-stimulated cells. Data represent the mean +/- S.E. of fold increase above baseline secretion in three separate experiments. *** = $p \leq .001$, * = $p \leq .05$.

Interleukin-10 secretion by B26 cells was increased significantly at 6r and 12 hr, but the levels at 12 hr were less than at 6 hr (Figure 3A). Similar responses were observed in Y17 cells (Figure 3B), though the magnitude was less than that of B26. Responses to stimulation in VT8 cells were higher at 6 hr, but not 12 hr (Figure 3C). For RT9 cells, IL-10 secretion was significantly increased by stimulation for 6 hr, but not 12 hr (Figure 3D). As was observed

with IL-6 production in response to stimulation, JOR cells produced very low levels of IL10 upon stimulation (Figure 3E).

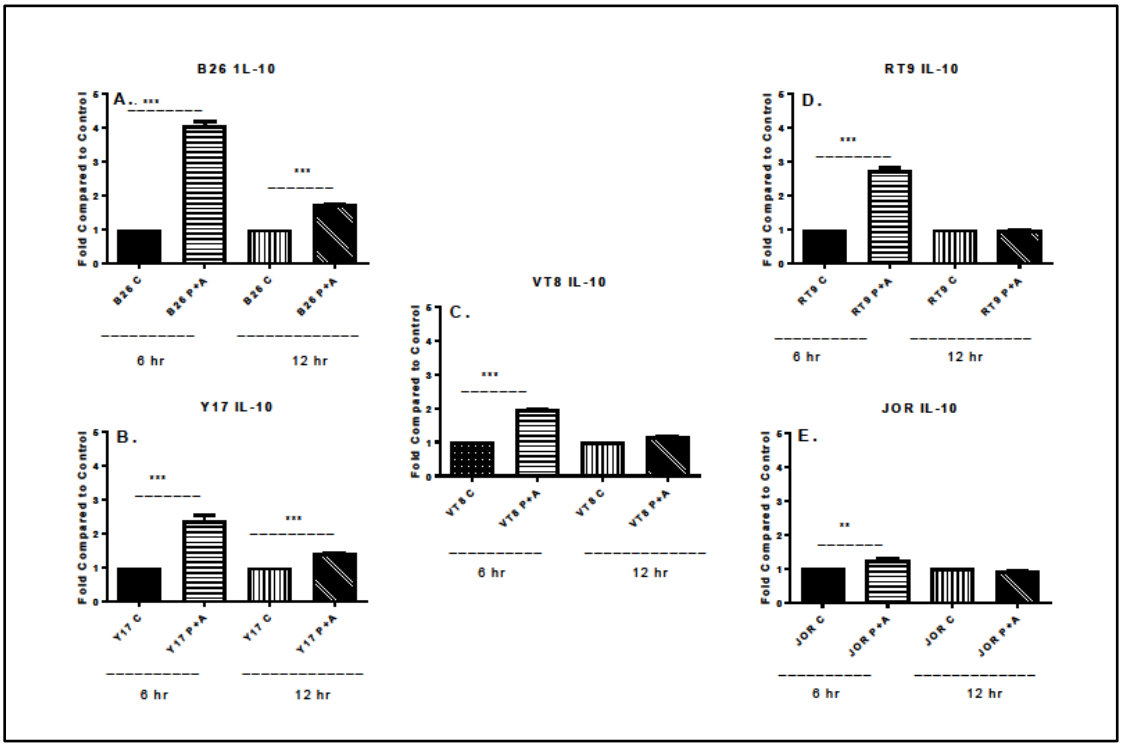

Figure 3. Interleuking-10 secretion by lymphoblast cell lines at 6 hr and 12 hr culture after stimulation with PMA + A23187. Cytokine levels were quantified by ELISA, and the data represent levels above non-stimulated cells. Data represent the mean +/- S.E. of fold increase above baseline secretion in three separate experiments. *** = $p \leq .001$, ** = $p \leq .01$.

Expression of RAG-1 and AHR in lymphoblast cell lines

Cellular expression of recombination activation gene-1, RAG-1, evident in all 5 cell lines with slight reduction in Y17 cells (Figure 4). These levels were as

high as can be seen in the mouse thymocyte lysate (Thy), and all bands were at the expected MW of approximately 135 kDa.

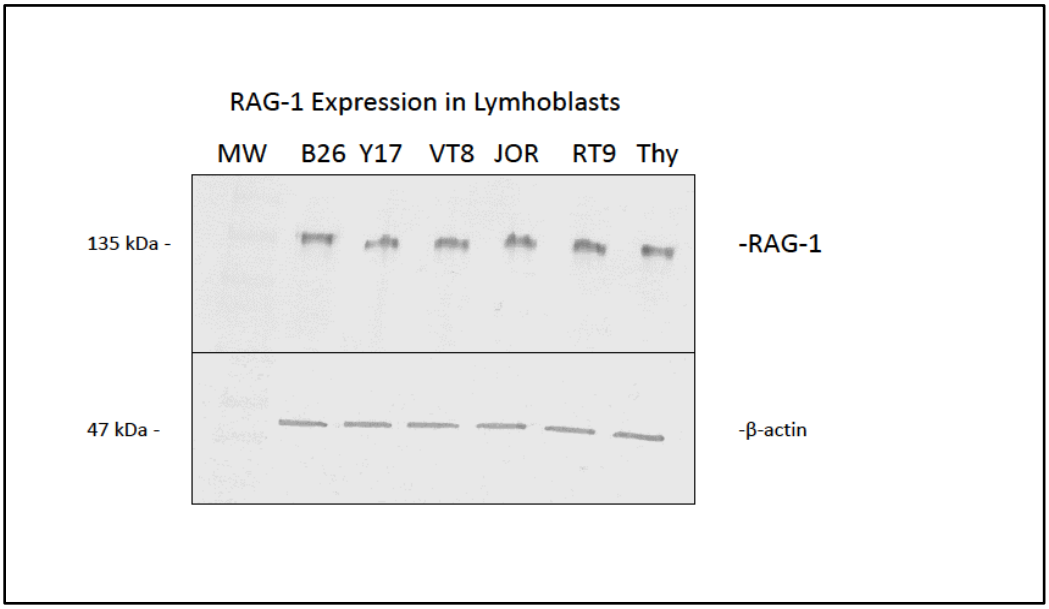

Figure 4. Expression of RAG-1 protein in whole cell lysates from lymphoblast cell lines. Thirty ug of lysate protein were subjected to SDS-PAGE and immunoblotting using a RAG-1-specific antibody. Equal amounts of protein were subjected SDS-PAGE and immunoblotting using a β-actin specific antibody. Whole mouse thymus tissue lysates (Thy) were used as a positive control for RAG-1

Cellular expression of the AHR was evident in all 5 cell lines, with a slight reduction in the Y17, and perhaps VT8 (Figure 5, Figure 6). These proteins were of the expected molecular weight of approximately 100 kDa, and were expressed at about the same level as lysates from the glioblastoma cell line (GLI).

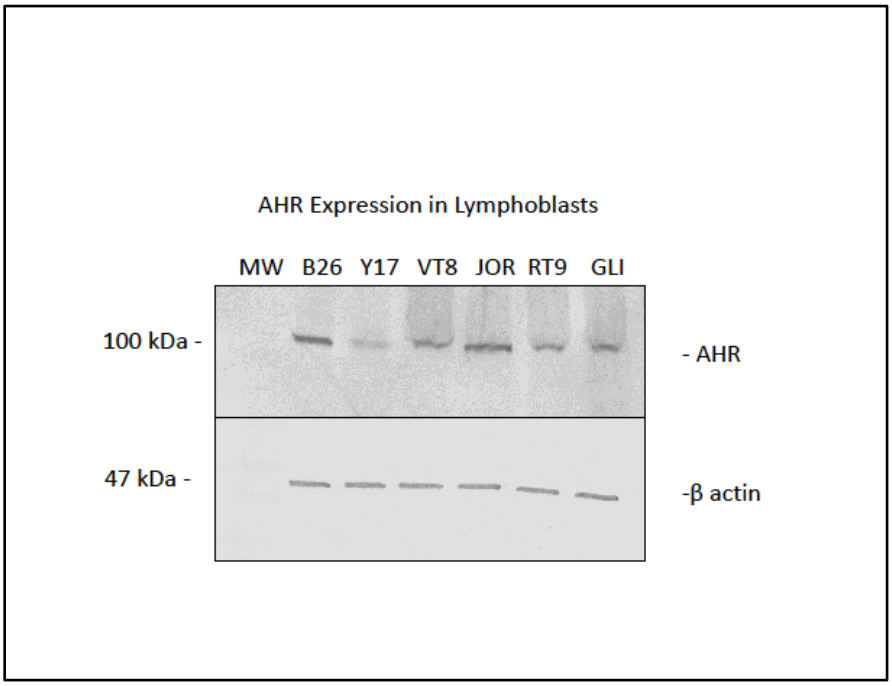

Figure 5. Expression of AHR protein in whole cell lysates from lymphoblast cell lines. Thirty ug of lysate protein were subjected to SDS-PAGE and immunoblotting using a AHR-specific antibody. Equal amounts of protein were subjected SDS-PAGE and immunoblotting using a β-actin specific antibody. Human glioblastoma T98G cell lysates (GLI) were used as a positive control for RAG-1

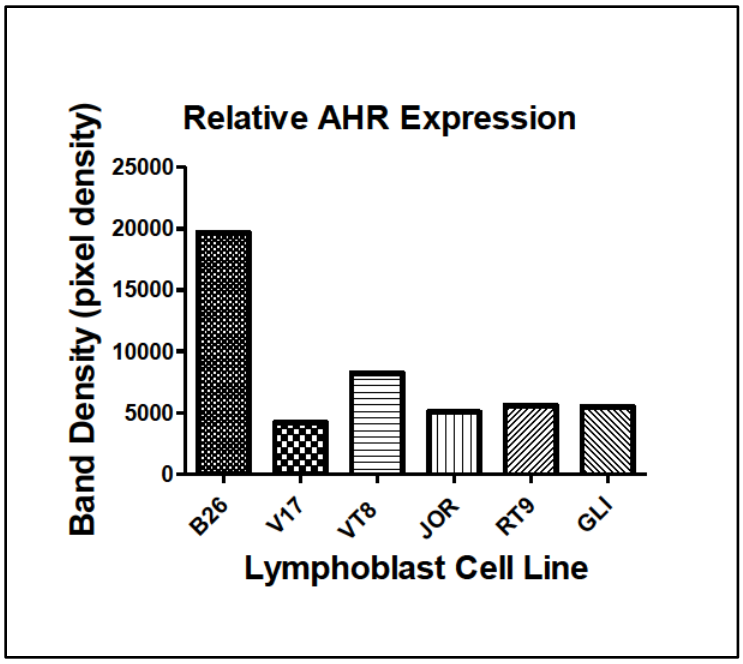

Figure 6. Band density of AHR expression in lymphoblasts. Bands density was determined using Image J software (NIH).

CYP1B1 and IDO2 expression as markers for the trypto-AHR pathway

In response to the endogenous AHR ligand FICZ, CYP1B1 induction was highest in B26 cells, and was significantly higher than the other four cells lines (Figure 6A). When examining for differences in CYP1B1 mRNA levels between all pair-wise comparisons, there we no differences between the V17, VT8, JOR, and RT9. Expression levels in VT8, JOR, and RT9 were roughly two-fold, with Y17 cells expressing close to 4 fold levels, and B26 cells expressing nearly twice that level.

In response to PMA + A2317 as a means to activate the cells to induce expression of IDO2, B26 cells responded more than the other four cell lines (Figure 6B). When examining for differences in IDO2 mRNA levels between all pair-wise comparisons, there we no differences between the V17, VT8, JOR, and RT9. Expression levels in Y17, VT8, JOR, and RT9 were roughly 3 – 4 fold, with B26 cell expressing nearly twice that level.

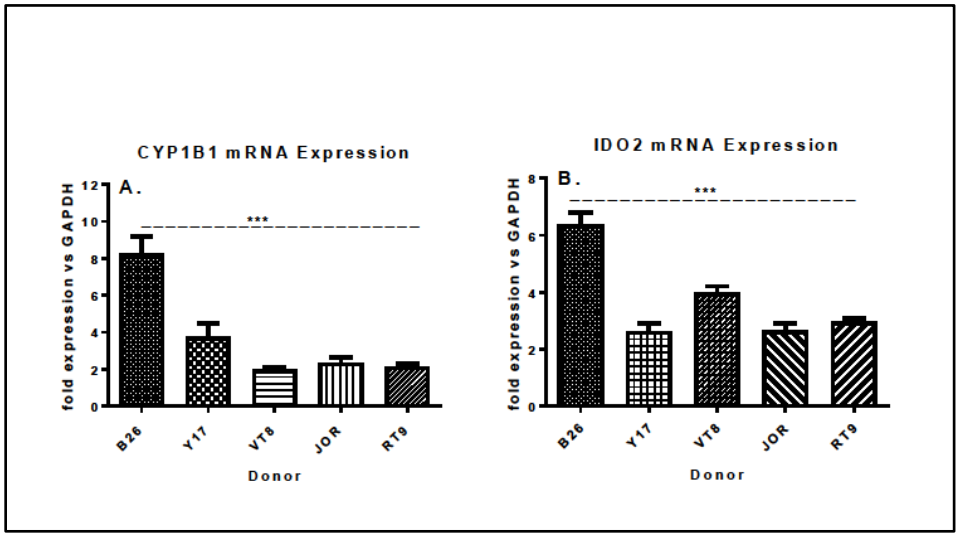

Figure 7. CYP1B1 mRNA induction in lymphoblast cells lines following 12 hr treatment with FICZ (A). IDO2 mRNA induction in lymphoblast cells lines following 12 hr treatment with PMA + A23187 (B). Data represent the mean +/- S.E. of fold increase over GAPDH mRNA expression in three separate experiments. *** represent $p \leq .001$.

Effects of tryptophan on expression of genes involved in tryptophan metabolism

A commercially available qPCR array platform for evaluating tryptophan metabolism was used as a screening approach to determine if treatment with tryptophan modulates changes in mRNA expression of genes involved in tryptophan metabolism in the lymphoblasts. The data provides an indication of relative magnitude of expression compared to DMSO controls and allows observation of relative changes in gene expression associated with inflammation, apoptosis, cell cycle control, and oxidative stress. Under these conditions, some of the lymphoblasts exhibit an increased expression of several mRNAs associated with activation, signaling, pro-inflammatory, and catabolism of tryptophan available on this array (Figure 1). For each mRNA evaluated on the array, treatment either decreased expression (more than 2-fold suppression), had no effect, or only slightly increased (2-fold or higher). Of note, B26 was the very high expresser for some genes involved in energy conversion/metabolism, melatonin synthesis, and genes involved biosynthesis of arginine (ACADM, AANAT, ACAT1, ACMSD, ALDH18A1, ARG2, ASL, and BHMT). For B26, all of these genes were significantly expressed (>10-fold). It can be noted that both Y17 and VT8, the parents of B26, had some of the same significant increases in some genes similar to B26, namely ACADM, ACAT1, ALDH18A1. Several mRNAs were intermediate in expression (>2-fold, <10-fold).

Gene name	Genbank #	Symbol	B26	VT8	Y17	JOR	F34	PO1	T48	GN12
Aminoadipate aminotransferase	NM_016228	AADAT	-1.70	-1.70	1.39	1.75	1.91	4.75	7.53	-1.55
Aralkylamine N-acetyltransferase	NM_001088	AANAT	17.13	10.87	1.03	-3.87	2.95	1.15	8.30	2.31
Acyl-CoA dehydrogenase medium chain	NM_000016	ACADM	13746.90	2947.08	-1.17	2.44	2.37	136.42	848.69	1.66
Acetyl-CoA acetyltransferase 1	NM_000019	ACAT1	140.28	19.32	-1.19	2.51	1.64	3.56	23.93	1.59
Aminocarboxymuconate semialdehyde decarboxylase	NM_138326	ACMSD	12.93	2.51	-5.36	-2.38	6.49	-1.68	12.29	1.48
Aldehyde dehydrogenase 9, family member A1	NM_000696	ALDH9A1	3.52	1.45	-1.07	-1.26	1.59	2.34	21.18	1.32
Aldehyde dehydrogenase 18, family member A1	NM_002860	ALDH18A1	11.83	11.24	-2.28	3.11	3.75	24.90	23.21	1.14
Amine oxidase, Cu containing 1	NM_001091	AOC1	-1.70	-1.70	1.39	45.14	1.91	1.92	-1.41	-1.55
Aldehyde oxidase 1	NM_001159	AOX1	-1.70	-3.85	1.39	1.28	-2.09	1.92	-1.41	-1.55
Arginase 2	NM_001172	ARG2	12.10	3.73	-1.07	1.53	1.88	-1.26	6.51	1.43
Argininosuccinate lyase	NM_000048	ASL	17.25	3.70	-1.22	1.28	1.88	-1.28	5.40	1.10
Betaine-homocysteine S-methyltransferase	NM_001713	BHMT	24.51	-1.60	-17.29	1.64	1.75	1.92	4.10	10.78
Catalase	NM_001752	CAT	1.61	-1.02	-1.48	-1.29	2.12	-2.31	4.26	1.15
CYP1B1	NM_000104	CYP1B1	7.58	5.56	-1.06	-3.34	-2.05	1.83	29.25	2.58
DOPA decarboxylase	NM_000790	DDC	1.06	-2.06	1.41	1.09	-1.42	3.88	-1.41	1.89
Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase	NM_001966	EHHADH	2.78	-4.13	-1.05	-1.27	2.48	3.03	7.43	1.57
Glutaryl-CoA dehydrogenase	NM_000159	GCDH	2.19	1.08	-1.05	1.51	1.49	-1.50	1.44	-1.02
3-hydroxyanthranilate 3,4-dioxygenase	NM_012205	HAAO	1.10	1.07	-1.49	1.52	1.28	-1.42	2.10	1.39
Indoleamine 2,3-dioxygenase 1	NM_002164	IDO1	-10.35	-1.70	1.39	-5.62	1.91	1.92	5.32	-1.55
Indolethylamine N-methyltransferase	NM_006774	INMT	2.39	-1.70	-7.24	-1.06	1.91	1.92	-6.46	-4.06
Kynurenine 3-monooxygenase	NM_003679	KMO	1.02	1.28	-3.92	-1.28	1.54	-2.12	2.75	-1.12
Kynureninase	NM_003937	KYNU	1.56	1.51	-3.12	1.36	1.03	-2.47	3.85	1.03
Monoamine oxidase B	NM_000898	MAOB	-1.70	-1.70	1.39	-1.70	1.91	1.92	-1.41	-1.55
Nitrilase family member 2	NM_020202	NIT2	1.53	1.45	-1.82	-1.70	-1.48	-1.60	29.33	2.39
Oxoglutarate dehydrogenase like	NM_018245	OGDHL	-1.16	1.68	2.24	-1.70	-1.19	2.31	1.02	-1.22
Tryptophan 2,3-dioxygenase 2	NM_005651	TDO2	-2.18	-1.70	1.39	-1.70	4.28	1.92	1.80	-1.55
Tryptophan hydroxylase 2	NM_173353	TPH2	-1.70	-1.70	-2.31	1.05	1.91	1.92	-1.41	-1.07
Tryptophanyl-tRNA synthetase	NM_004184	WARS	1.03	1.53	-1.11	-1.15	1.10	1.43	2.36	1.31

Table 2 shows an array outlining fold change in genes involved in tryptophan metabolism for all cell lines using a commercial qPCR Amino Acid Metabolism Array

DISCUSSION

In this study I found that lymphoblasts from ASD patient B26 stand out from the other 4 cell lines and may provide a wealth of information in the fields of inflammation, AHR biology, toxicology, mitochondrial functions, and possibly others, including ASD. This cell line secretes low basal levels of IL-6 compared to other lines but responded very well to stimulation with PMA + A23187 by secreting more IL-6 than the other lines. This observation suggests that PKC, NFkB, AP-1 and other signaling components are intact. Though not examined in this study, studies examining fibroblast mitochondrial numbers are much higher in B26 than the other lines, and if this holds true for B26 lymphoblasts, then that may explain the rapid growth of this line compared to the others. The control donor cells secreted high background levels of IL-6 in comparison to B26 and his two parents, and may be useful in generating IL-6 supernatants to support hybridomas during the production of monoclonal antibodies, since mouse cells respond to human IL-6 [72]. The high levels of CYP1B1 induction by the endogenous AHR ligand FICZ suggests that AHR signaling in B26 cells is not altered by the EBV-transformation process, and this cell line may prove useful in future studies examining AHR biology. This study can draw no conclusions that would suggest the other 4 cell lines have impaired AHR signaling, but a few of the lines expressed less AHR protein than did B26. All 5 lines expressed levels of RAG-1 comparable to fresh mouse thymocytes, which are a rich source of RAG-1, suggesting that RAG-1 has been reactivated during the EBV-

transformation process. During normal B-cell development, RAG-1 (and RAG-2) are expressed at two key check points to generate a functional heavy chain, followed by the generation of a functional light chain, then the expression is turned off in a mature B-cell. It may be that the transformation process drives the B-cell back towards a pre-B-cell stage in which RAG-1 is re-expressed. RAG-1 may also be re-expressed during receptor editing when B-cell has the opportunity to generate a new light chain if the original light chain recognizes “self”. These cell lines may provide a model for RAG-1 genetics. To my knowledge, there is no relationship between RAG-1 expression and ASD.

In this study it was found that key genes involved in tryptophan metabolism are not altered in patient B26, either of its parents, nor the two donor controls. The mother of B26, VT8, was likewise responsive to tryptophan in terms of CYP1B1 induction. The array data shows that several genes involved in mitochondrial function were very responsive to tryptophan treatment in B26, and similarly in the mother, Y 17. Genes not only involved in energy metabolism, but also inflammation and different pathways stemming from tryptophan metabolism (serotonin and melatonin pathways) were affected. Although not yet published, studies in Dr. William Baldwin’s lab at Clemson University show that fibroblasts from B26 have very high numbers of mitochondria compared to other patients and donors, but B26 fibroblasts did not demonstrate ample repair mechanisms during hydrogen peroxide oxidative stress associated with mitochondria, and mitochondrial repair. Similar observations were made in Y17, the mother of B26.

CYP1B1 and IDO2 are two genes expressed in tryptophan metabolism pathways, though the commercial array did not have IDO2 as one of the primer sets. CYP1B1 is expressed when the AHR is activated, as seen with treatment with FICZ in the qPCR experiments – FICZ us a potent endogenous ligand for the AHR. IDO2 is expressed during the catabolism of tryptophan in a variety of tissues, and especially activated lymphocytes, leading to the formation of intermediate metabolites, several of which are known ligands for the AHR. An increase in these two genes in ASD individuals could point to a dysfunctional metabolism of tryptophan and its metabolites. Only one severely autistic patient is represented in my study, but CYP1B1 and IDO2 may be expressed at higher levels in people with ASD because tryptophan is broken down rapidly, therefore favoring the AHR and being infrequently utilized in other pathways, such as the serotonin pathway. Kynurenine plays an important role in preventing autoimmune responses by generating regulatory T-cells through the activation of AHR. In ASD, it seems that tryptophan is directed away from the kynurenine pathway and results in a decreased production of kynurenine [73]. Low kynurenine is associated with a lower than normal regulatory T- cell production (Treg) and a higher risk of developing autoimmunity. This is demonstrated by the increased catabolism of tryptophan in ASD patients compared to donor controls. However, there is no current evidence that ASD is an autoimmune disease, but there may be a link, and my results with B26 may lead to further studies on this subject.

There is no rapid laboratory test to diagnose ASD, although several biomarkers have been associated with it – namely altered tryptophan metabolism. My study may provide a few biomarkers, such as B-cell activation by PMA + A23187, peripheral lymphocyte CYP1B1 induction by FICZ, and IDO2 induction by PMA + A23187. It may be too cumbersome to use a commercial tryptophan metabolism array such as the one I used, but perhaps select genes associated with mitochondrial function would be appropriate.

The cause of ASD is not known and likely involves interactions between genetic factors and environmental factors. Previous articles have shown that there is a link between an over-expression of IL-6 and increased severity in ASD symptoms. IL-6 is ordinarily expressed at reasonably low levels in the brain, but in the presence of injury or inflammation, IL-6 is elevated in spinal fluid as well as brain homogenates [74]. In a study done by Wei, H. et al., it was shown that chronic overexpression of IL-6 in mice causes anatomical and physiological changes in the brain, leading to disorders such as ASD. IL-10 is a well-known anti-inflammatory cytokine, and increased levels could point to a negative feedback loop type process. As levels of IL-6 increase, levels of IL-10 increase to try and lower IL-6. In this study, levels of IL-6 and IL-10 in non-stimulated lymphoblasts were observed. It seems as though IL-6 levels are lowest in B26 and the parents of B26, and perhaps because IL-10 helps to blunt inflammation, therefore decreasing IL-6 levels.

Typically, many children with ASD are not diagnosed until the age of 2 or 3, meaning that the window to intervene early is essentially closed before a diagnosis is even made [61]. Even then, diagnosis depends on parent questioning, and seems subjective. As mentioned before, B26, the severely autistic child, has abnormalities on chromosome 10 and 15, both inherited from the parents Y17 and VT8. It is important to note that although Y17 (mother) has not been placed on the the autism spectrum, she had very similar responses to tryptophan as B26 (the child), as seen in the IL-6 and IL-10 figures. All of these data may point to more than a genetic association between specific chromosomal deletions and ASD. It was previously mentioned that there are thousands of submicroscopic structural variants covering up to roughly 18% of the human reference DNA sequence, which means that no one micro deletion or duplication points to ASD. It does mean, though, that there could be a possible way to diagnose ASD with genetic testing when the child is still very young, especially in families who have already had a child or children with ASD. My assays and methods may be used in combination with analysis of genes involved in mitochondrial function to shed light on the possibility of an earlier diagnosis.

FUTURE DIRECTION

Moving forward from this study, a larger pool of study subjects (lymphoblast lines) would be beneficial, as there were only five subjects treated in this study. This would be especially helpful in performing another commercial amino acid array, or even RNAseq, as more cell lines would aide in possible links between these genes and severity of ASD. Although it was beneficial to have samples from the parents of B26, because it assisted in seeing a possible role of genetics in the development of ASD, it would have been more helpful to have more samples from patients with ASD of varying severity. As mentioned previously in this study, there was only one patient with severe ASD. ASD is a spectrum, as the name suggests, and being able to connect expression of genes and proteins of interest to the known severity of patients would have facilitated data to possibly be able to not only diagnose ASD with a simple blood test, but also the severity of the condition from an earlier age than when ASD is currently diagnosed.

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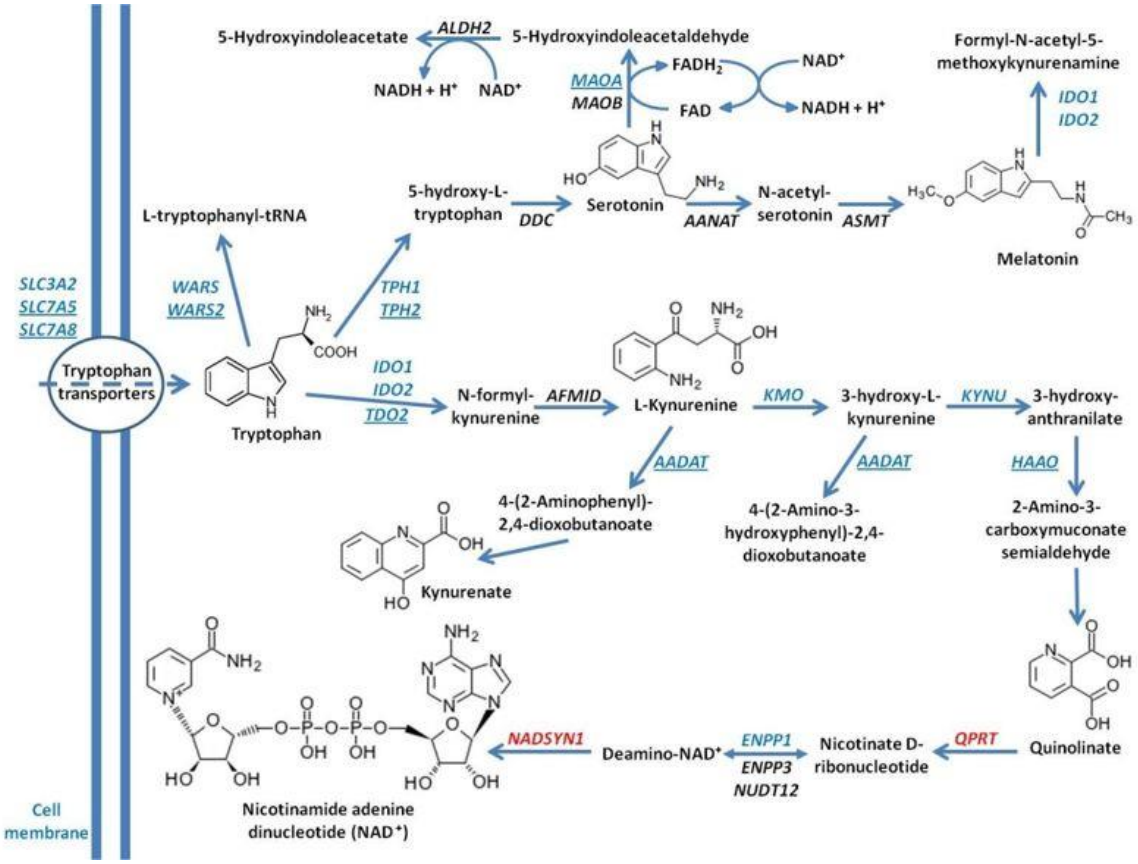

Illustration 1 outlines the main intracellular pathways involving tryptophan, as well as the genes involved in tryptophan metabolism

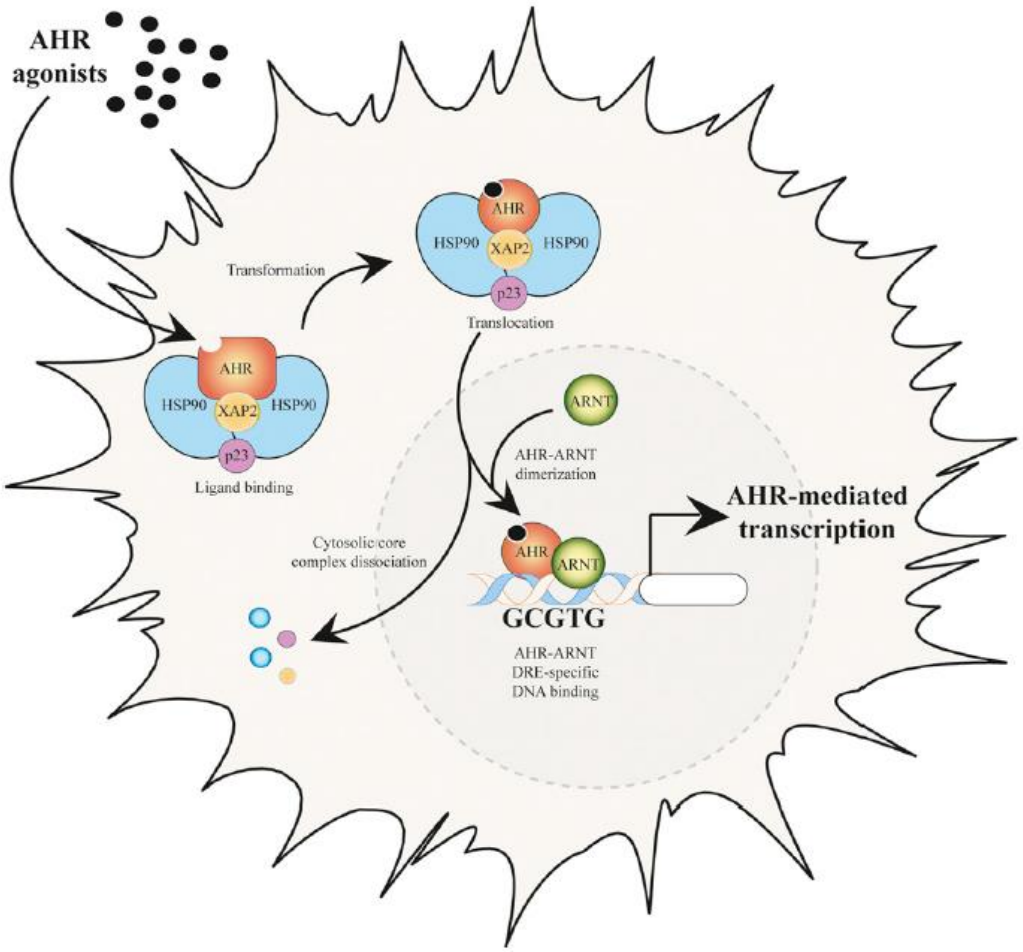

Illustration 2 summaries the pathway of AhR-mediated transcription after binding. The AHR and ligand are displaced from the cytoplasm to the nucleus after binding, leading to transcription of both CYP1B1 and IDO2 in this study

APPENDIX A

Some genes highly expressed by B26, and the mother, Y17

Acetyl-CoA Acetyltransferase (ACAT1): This gene encodes an enzyme located in the mitochondria that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA. The ACAT enzyme is activated by potassium ions binding near the CoA binding site and the catalytic site. This binding causes a structural change in the active site loop. Additionally, this enzyme is able to use 2-methyl-branched acetoacetyl-CoA as a substrate, making it a unique thiolase. ACAT1 is regulated at both transcriptional and translational levels. ACAT1's expression is promoted transcriptionally by leptin, angiotensin II, and insulin in human monocytes. Insulin-mediated regulation also involves ERK, p38MAPK, and JNK signaling pathways.

Aralkylamine N-acetyltransferase (AANAT): The protein encoded by this gene belongs to the acetyltransferase superfamily. It is the penultimate enzyme in melatonin synthesis and controls the night/day rhythm in melatonin production in the vertebrate pineal gland. Melatonin is essential for the function of the circadian clock that influences activity and sleep. This enzyme is regulated by cAMP-dependent phosphorylation that promotes its interaction with 14-3-3 proteins and thus protects the enzyme against proteasomal degradation.

Aminocarboxymuconate Semialdehyde Decarboxylase (ACMSD): The neuronal excitotoxin quinolinate is an intermediate in the de novo synthesis pathway of NAD from tryptophan, and has been implicated in the pathogenesis of several neurodegenerative disorders. Quinolinate is derived from alpha-amino-beta-carboxy-muconate-epsilon-semialdehyde (ACMS). ACMSD (ACMS decarboxylase; EC 4.1.1.45) can divert ACMS to a benign catabolite and thus prevent the accumulation of quinolinate from ACMS.

4-trimethylaminobutyraldehyde Dehydrogenase (ALDH9A1): This protein belongs to the aldehyde dehydrogenase family of proteins. It has a high activity for oxidation of gamma-aminobutyraldehyde and other amino aldehydes. The enzyme catalyzes the dehydrogenation of gamma-aminobutyraldehyde to gamma-aminobutyric acid (GABA). This isozyme is a tetramer of identical 54-kD subunits.

Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase (EHHADH): his protein is involved in the pathway fatty acid beta-oxidation, which is part of Lipid metabolism.

Amine Oxidase, Copper Containing 1 (AOC1): amine oxidase, copper containing 1.This gene encodes a metal-binding membrane glycoprotein that oxidatively deaminates putrescine, histamine, and related compounds. The

encoded protein is inhibited by amiloride, a diuretic that acts by closing epithelial sodium ion channels. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene.

Acyl-CoA Dehydrogenase Medium Chain (ACADM): This enzyme is essential for converting these fatty acids to energy, especially during periods without food (fasting). The ACADM enzyme functions in mitochondria, the energy-producing centers within cells. It is found in the mitochondria of several types of tissues, particularly the liver.

Aldehyde Dehydrogenase 18 Family Member A1 (ALDH18A1): Delta-1-pyrroline-5-carboxylate synthetase (P5CS) is an enzyme that in humans is encoded by the *ALDH18A1* gene. This gene is a member of the aldehyde dehydrogenase family and encodes a bifunctional ATP- and NADPH-dependent mitochondrial enzyme with both gamma-glutamyl kinase and gamma glutamyl phosphatereductase activities. The encoded protein catalyzes the reduction of glutamate to delta1-pyrroline-5-carboxylate, a critical step in the de novo biosynthesis of proline, ornithine and arginine. Mutations in this gene lead to hyperammonemia, hypoornithinemia, hypocitrullinemia, hypoargininemia and hypoprolinemia and may be associated with neurodegeneration, cataracts, and connective tissue diseases. Alternatively spliced transcript variants, encoding different isoforms, have been described for this gene.

Aldehyde Dehydrogenase 4 Family Member A1 (ALDH4A1): This protein belongs to the aldehyde dehydrogenase family of proteins. This enzyme is a mitochondrial matrix NAD-dependent dehydrogenase which catalyzes the second step of the proline degradation pathway, converting pyrroline-5-carboxylate to glutamate. Deficiency of this enzyme is associated with type II hyperprolinemia, an autosomal recessive disorder characterized by accumulation of delta-1-pyrroline-5-carboxylate (P5C) and proline. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene.

Methylthioribulose 1-phosphate Dehydratase (APIP): Involved in the methionine salvage pathway. APIP deficiency is associated with cell death and cancer.

Arginase 2 (ARG2): Arginase catalyzes the hydrolysis of arginine to ornithine and urea. At least two isoforms of mammalian arginase exists (types I and II) which differ in their tissue distribution, subcellular localization, immunologic crossreactivity and physiologic function. The type II isoform encoded by this gene, is located in the mitochondria and expressed in extra-hepatic tissues, especially kidney. The physiologic role of this isoform is poorly understood; it is thought to play a role in nitric oxide and polyamine metabolism. Transcript variants of the type II gene resulting from the use of alternative polyadenylation sites have been described

Argininosuccinate Lyase (ASL): An enzyme that catalyzes the reversible breakdown of argininosuccinate (ASA) producing the amino acid arginine and dicarboxylic acid fumarate. Located in liver cytosol, ASL is the fourth enzyme of the urea cycle and involved in the biosynthesis of arginine in all species and the production of urea in ureotelicspecies. Mutations in ASL, resulting low activity of the enzyme, increase levels of urea in the body and result in various side effects.

Argininosuccinate synthetase (ASS1): An enzyme that in humans is encoded by the *ASS1* gene. The protein encoded by this gene catalyzes the penultimate step of the arginine biosynthetic pathway. There are approximately 10 to 14 copies of this gene including the pseudogenes scattered across the human genome, among which the one located on chromosome 9 appears to be the only functional gene for argininosuccinate synthetase. Two transcript variants encoding the same protein have been found for this gene.

3-methylglutaconyl-CoA hydratase (AUH): An enzyme encoded by the *AUH* gene on chromosome 19. It is a member of the enoyl-CoA hydratase/isomerase superfamily, but it is the only member of that family that is able to bind to RNA. Not only does it bind to RNA, AUH has also been observed to be involved in the metabolic enzymatic activity, making it a dual role protein. Mutations of this gene have been found to cause a disease called 3-Methylglutaconic Acuduria Type 1.

Branched Chain Amino Acid Transaminase 2 (BCAT2): This gene encodes a branched chain aminotransferase found in mitochondria. The encoded protein forms a dimer that catalyzes the first step in the production of the branched chain amino acids leucine, isoleucine, and valine. Multiple transcript variants encoding different isoforms have been found for this gene.

Branched Chain Keto Acid Dehydrogenase E1, Alpha Polypeptide (BCKDHA): 2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial is an enzyme that in humans is encoded by the *BCKDHA* gene. The second major step in the catabolism of the branched-chain amino acids, isoleucine, leucine, and valine, is catalyzed by the branched-chain alpha-keto acid dehydrogenase complex (BCKD; EC 1.2.4.4), an inner-mitochondrial enzyme complex that consists of 3 catalytic components:

Betaine-homocysteine S-methyltransferase (BHMT): This gene encodes a cytosolic enzyme that catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively. Defects in this gene could lead to hyperhomocysteinemia, but such a defect has not yet been observed.

Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase, and Dihydroorotase (CAD): Trifunctional multi-domain enzyme involved in the first three steps of pyrimidine biosynthesis. De-novo synthesis starts

with cytosoliccarbamoylphosphate synthetase II which uses glutamine, carbon dioxide and ATP. This enzyme is inhibited by uridine triphosphate (feedback inhibition).

Cystathionine-β-synthase (CBS): An enzyme (EC4.2.1.22) that in humans is encoded by the *CBS* gene. It catalyzes the first step of the transsulfuration pathway, from homocysteine to cystathionine: L-serine + L-homocysteine L-cystathionine + H_2O. CBS uses the cofactor pyridoxal-phosphate (PLP) and can be allosterically regulated by effectors such as the ubiquitous cofactor S-adenosyl-L-methionine (adoMet). This enzyme belongs to the family of lyases, to be specific, the hydro-lyases, which cleave carbon-oxygen bonds. CBS is a multidomain enzyme composed of an N-terminal enzymatic domain and two CBS domains. The CBS gene is the most common locus for mutations associated with homocystinuria.

Carbamoyl-phosphate Synthase 1 (CPS1): The mitochondrial enzyme encoded by this gene catalyzes synthesis of carbamoyl phosphate from ammonia and bicarbonate. This reaction is the first committed step of the urea cycle, which is important in the removal of excess urea from cells. The encoded protein may also represent a core mitochondrial nucleoid protein. Three transcript variants encoding different isoforms have been found for this gene. The shortest isoform may not be localized to the mitochondrion. Mutations in this

gene have been associated with carbamoyl phosphate synthetase deficiency, susceptibility to persistent pulmonary hypertension, and susceptibility to venoocclusive disease after bone marrow transplantation

Cystathionine gamma-lyase (CTH): An enzyme which breaks down cystathionine into cysteine, α-ketobutyrate, and ammonia. Pyridoxal phosphate is a prosthetic group of this enzyme. Cystathionine gamma-lyase also catalyses the following elimination reactions: L-homoserine to form H_2O, NH_3 and 2-oxobutanoate; L-cystine, producing thiocysteine, pyruvate and NH_3; L-cysteine producing pyruvate, NH_3 and H_2S. In some bacteria and mammals, including humans, this enzyme takes part in generating hydrogen sulfide. Hydrogen sulfide is one of a few gases that was recently discovered to have a role in cell signaling in the body.

β_2 microglobulin (B2M): A component of MHC class I molecules, which are present on all nucleated cells (excludes red blood cells). In humans, the β_2 microglobulin protein is encoded by the *B2M* gene.