# DYSREGULATED ERK SIGNAL PATHWAY AND IMMUNE PROFILES IN FRAGILE X SYNDROME

BY

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DISSERTATION

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**ABSTRACT**

Fragile X Syndrome (FXS) is the leading cause of inherited mental retardation, and the most common identified genetic cause of autism. Lack of production of the Fragile X Mental Retardation Protein (FMRP) leads to changes in dendritic morphology and resultant cognitive and behavioral manifestations characteristic of individuals with FXS. FMRP is an RNA-binding protein that is believed to regulate the translation of a large number of other proteins, leading to a complex and variable set of symptoms in FXS. In a mouse model of FXS, we previously observed delayed initiation of synaptically localized protein synthesis in response to neurotransmitter stimulation, as compared to wild-type mice. We now likewise have observed delayed early-phase phosphorylation of extracellular-signal regulated kinase (ERK), a nodal point for cell signaling cascades, in both neurons and thymocytes of *fmr-1* KO mice. We further reported that early-phase kinetics of ERK activation in lymphocytes from human peripheral blood is delayed in a cohort of individuals with FXS, relative to normal controls, suggesting a potential biomarker to measure metabolic status of disease for individuals with FXS. Furthermore, dysregulated phosphatases, especially Protein phosphatase 2A (PP2A) may account for the delay in ERK activation.

FXS and immune dysregulation is an emerging area in FXS research. We hypothesize that immune cells from FXS patients may have different gene expression and protein profiles. We first analyzed genome-wide microarray data from FXS lymphoblastoid cell, and found several immune gene sets are differentially expressed in FXS patients. We further reported that the cytokine profiles and cytokines activation

profiles are dysregulated in FXS patients. These results could be used as potential cellular markers for FXS patients.

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# CHAPTER 1

# INTRODUCTION

## Etiology of Fragile X Syndrome

Fragile X syndrome (FXS) is the most common cause of inherited mental retardation, with an estimated incidence of 1/4000 males and 1/6000 females (Brown, 1996; Turner et al., 1996; Crawford et al., 2002). Clinically FXS is characterized by various physical signs such as long face, large ears and macro-orchidism (Davies, 1989; Hagerman and Hagerman, 2002). FXS is often accompanied by seizures, intellectual disability, cognitive disabilities ranging from mild to severe (Hagerman and Sobesky, 1989; Hagerman and Hagerman, 2002; Hatton et al., 2002); notably, about 46–67% of FXS patients have autism spectrum disorders (ASD) or "autistic-like" behaviors including repetitive behavior, decreased attention, and poor eye contact (Clifford et al., 2007; Bailey et al., 2008).

In neuronal anatomy studies, it is now commonly accepted that FXS affects dendritic spine maturation. Several independent laboratories have shown that spine length and density is increased in *fmr-1* Knockout (KO) brain neurons compared to those in Wild-Type (WT) mice (Comery et al., 1997; Irwin et al., 2000). This finding is consistent with the findings in humans (Irwin, Patel et al. 2001) and further supports the hypothesis of a maturation and pruning defect.(Galvez et al., 2003; Galvez and Greenough, 2005)

**FMRP and FMRP cargoes**

FXS is caused by a mutation in the *Fmr1* gene on the X chromosome (localized at Xq27.3). In most cases, the coding sequence itself is not changed, but a trinuclotide (CGG) repeat in the 5'-untranslated region (5'-UTR) is expanded (Fu et al., 1991). This expansion causes hypermethylation of CpG islands (Verkerk et al., 1991) and partially or fully silences *Fmr1* gene expression (McConkie-Rosell et al., 1993; Verheij et al., 1993).

The *Fmr1* gene is widely expressed in various tissues. In human and murine embryos, high expression levels of *Fmr1* are found in the brain, as well as non-neural tissues like the testis, eye and gonad (Mandel and Heitz, 1992). In adult, high levels of *Fmr1* transcripts are found in various brain regions and testis/ovaries, as well as immune tissues like thymus and spleen (Pieretti et al., 1991; Hagerman and Hagerman, 2002).

In the early 1990's, researchers identified the gene product of *Fmr1*, Fragile X mental retardation protein (FMRP) (Pieretti et al., 1991), opening a new chapter of FXS research. Intensive molecular analysis revealed that FMRP is a RNA binding protein. It contains two K Homology (KH) domains and an Arginine-glycine Rich (RGG) box, both of which are known to be RNA binding motifs (Ashley et al., 1993; Gibson et al., 1993), and FMRP has been shown to be associated with mRNP complexes in actively translating polyribosomes (Eberhart et al., 1996; Ceman et al., 2003; Khandjian et al., 2004; Stefani et al., 2004). Further studies showed FMRP can interact with RNA containing U-rich (Sung et al., 2000; Chen et al., 2003) or G-quartet (Darnell et al., 2001; Schaeffer et al., 2001) structures. FMRP also has nuclear localization and export signals (Bardoni et al.,

1997), suggesting that it can carry mRNAs and shuttle through nuclear pores. *In vitro* studies showed that FMRP can bind 4% of total human fetal brain mRNAs, suggesting the binding is broad but also selective (Brown et al., 1998).

Ever since FMRP's gene structure was revealed, there are numerous laboratories trying to identify the mRNA targets (cargoes) of FMRP. Brown and colleagues were the first to analyze the mRNAs associated with FMRP-mRNP on a large scale. They identified 432 mRNAs from mouse brain and 251 mRNAs from FXS patient lymphoblast cells by immunoprecipitation. Many of these mRNAs encode proteins important for neuronal cell signaling (Brown et al., 2001). Two years after Brown's work, Miyashiro et al performed an *in situ* approach using antibody-positioned RNA amplification to identify the mRNAs associated with FMRP in intact neuronal cultures (Miyashiro et al., 2003). In this work, most of the candidates were verified by UV crosslinking and filter binding assay. On Miyashiro's FMRP cargo list, some of the cargo mRNAs are involved in cell signaling /communications (Phosphoinositide 3-kinase(PI3K), some are involved in cell structure and motility (tubulin, Kinesin-like protein), but the majority of the FMRP cargos are actively involved in neuronal functions. Recent work by Darnell et al. 2011 revisited the question. By binding the FMRP cargo mRNAs with UV crosslinking-IP (CLIP) and followed by high-throughput sequencing (HITS-CLIP), Darnel was able to identify a stringent set of 842 FMRP targets from mouse brain tissues. The FMRP cargo mRNAs were enriched in neuronal and synaptic transmission and regulation of synaptic signaling (Darnell et al., 2011).

Although these results have some variances due to the difference in methodology, cell types, threshold to choose or discard a candidate etc., all indicate the interaction between FMRP and the cargo mRNAs could be important for regulating the distribution or abundance of proteins in cells, and crucial for neuronal functions.

**FMRP and translational regulation**

More recent research studies of FMRP were focused on the active role FMRP plays in FXS. It is now commonly accepted that FMRP is an mRNA transporter and a translational regulator.

Under basal conditions, FMRP acts as a global translation repressor. *In vitro*, FMRP inhibits translation of various mRNAs at nanomolar concentrations in both rabbit reticulocyte lysate (RLL) and microinjected *Xenopus laevis* oocytes (Laggerbauer et al., 2001). A general consequence of FMRP deficiency *in vivo* is excess global synthesis of proteins. This result is confirmed by elevated protein synthesis observed in KO mice total cerebral cortex (Qin et al., 2005).

Importantly, the influence of FMRP on specific protein levels may be either positive or negative and is subject to subcellular localization. For instance, Wang et al reported that FMRP down-regulates (or inhibits during early development) the synthesis of myelin basic protein (MBP) in oligodendrocytes (Wang et al., 2004). Li et al reported that the AMPA receptor subunit, GluR1, was decreased in the cortical synapses, but not in the hippocampus or cerebellum, of *fmr-1* KO mice (Li et al., 2002). D'Hulst et al demonstrated a decreased expression of the δ-subunit of the $GABA_A$ receptor in the

cortex, but not cerebellum of *fmr-1* KO mice (D'Hulst et al., 2006). Selby et al reported that trkB receptors are up-regulated in the cell bodies of parvalbumin-expressing hippocampal interneurons in KO mice, whereas they are down-regulated in apical dendrites of the KO as compared to WT (Selby et al., 2007). In Miyashiro's report, it was shown that synaptic protein levels were often different from total brain tissue levels (Miyashiro et al., 2003). These data demonstrated that FMRP may be able to down-regulate some proteins and up-regulate others, in a location-specific manner; suggesting that FMRP plays a complex and dynamic role in protein translation.

In 1997, Weiler et al reported that FMRP is translated near synapses in response to neurotransmitter activation (Weiler et al., 1997). Later they showed that synaptic stimulation induces strong protein synthesis including FMRP; in KO mice, this stimulation-induced translation at synapses is reduced or absent, suggesting FMRP is actively involved in local protein translation initiation in response to synaptic activation (Weiler et al., 2004). This model is further supported by research from other laboratories. For example, FMRP has been demonstrated to bind its own encoding *fmr1* mRNA. Glutamatergic signals increase FMRP and *fmr1* mRNA levels in dendrites, as well as *fmr1* containing granule trafficking (Antar et al., 2004). Todd et al. found that group I mGluR agonist drives the synthesis of both FMRP and the synaptic protein PSD-95 in cortical cultures but not in cultures from KO mice (Todd et al., 2003). Kao also reported CaMKIIα mRNA is delivered and translated in dendritic spines in response to group I mGluR stimulation in WT but not in KO neurons (Kao et al., 2010).

Taking these data together in a theoretical framework, it is believed that FMRP, like many RNA-binding proteins, acts as a translational repressor under unstimulated conditions; upon synaptic stimulation, FMRP mediates spatial mRNA transportation for local protein synthesis. In KO animals, at basal conditions, the translation repression is absent, and excess of proteins may be one reason for abnormal spine morphologies. However, under stimulated conditions, the directional transportation of the mRNA-containing granules and translational activation of the mRNAs is disrupted due to the loss of FMRP, and presumably, this disruption eventually affects synaptic plasticity and the neurological dysfunctions typical of the FXS (Weiler et al., 1997; Miyashiro et al., 2003; Weiler et al., 2004; Bagni and Greenough, 2005; Davidovic, 2005; Weiler, 2005).

In addition to the model mentioned above, there is strong evidence that FMRP phosphorylation affects its translational role. Phosphorylated FMRP cosediments with stalled ribosomes, whereas nonphosphorylated FMRP associates with actively translating ribosomes (Ceman et al., 2003). Narayanan et al further reported that upon mGluR stimulation, PP2A is rapidly activated and dephosphorylates FMRP, promoting a short period of protein translation activity. Extended mGluR activation then inactivates PP2A, and allows FMRP to return to its phosphorylated state (Narayanan et al., 2007).

**Lymphocytes ERK activation as a biomarker for FXS treatment**

In the mammalian brain, two forms of synaptic plasticity, Long-term potentiation (LTP) and Long-term depression (LTD), are used as models for learning and memory. Both processes require protein synthesis in response to electrical or neurotransmitter

stimulation (Malenka and Bear, 2004). Response to the agonist glutamate, based on metabotropic glutamate receptors, has been extensively studied. In FXS patients or KO mice, the delicate control of protein translation is altered, giving rise to many features of the FXS phenotype (Weiler et al., 1997; Weiler et al., 2004).

Most of the therapeutic agents developed for FXS treatment are targeted at repairing the dysregulated mGluR pathways (Levenga et al., 2010). However, testing the pharmaceutical efficacies on FXS patients is still challenging. Most of the behavior rating scales are subjective, along with the facts that many FXS patients have speech and language disorders as well as autistic-like behaviors; evaluations are mostly based on the clinicians' knowledge and experience. Clinical trials often involve a large amount of patients across multiple clinical sites, and the results are usually not sensitive. There is a clear need for a non-invasive, objective test for pharmaceutical evaluation in FXS clinical research.

Blood samples are easily accessible from patients and can be a desirable peripheral source for clinical studies. In fact, blood samples have been used to monitor drug effects in several psychiatric clinical trials including schizophrenia (Gama et al., 2007) , depression (Binder et al., 2004; Aydemir et al., 2006) and autism (Miyazaki et al., 2004; Hashimoto et al., 2006).

Evidence for the existence of glutamate receptors on immune cells emerged in the 1990s (Kostanyan et al., 1997). Several independent laboratories reported the expression of mGluRs in thymocytes and circulating lymphocytes. Storto et al reported

the existence of group I and II mGluRs in murine thymocytes, their results showed mGluR1 was present in immature CD4-/CD8- double-negative thymocytes, whereas mGluR5 was detected in more mature CD4-/CD8+ and CD4+/CD8- single-positive cells. mGluRs expressed in both thymic cell subsets were functionally active (Storto et al., 2000). In human lymphocytes, Pacheco et al reported that mGluR5 was expressed constitutively in T cells, whereas mGluR1 expression was induced after formation of the T-cell receptor/CD3 complex (Pacheco et al., 2004). All those results suggested possible mGluR signaling in lymphocytes.

The exact function of mGluRs on lymphocytes is still not clear. Current evidence suggests that glutamate might be used an immunotransmitter as well as a neurotransmitter. mGluRs expressed on lymphocytes cell surfaces could be involved in complex immune regulation, and mGluRs signaling may have a costimulatory role in T cell activation (Boldyrev et al., 2005).

The mGluR signaling in synapses and lymphocytes is quite similar (figure 1.1). Stimulation of mGluRs leads to G protein activation of phospholipase C (PLC) (Abdul-Ghani et al., 1996), splitting membrane phosphatidyl inositol (PI) into diacyl glycerol (DAG) and inositol triphosphate (IP3), which in turn releases $Ca^{2+}$ from intracellular stores (Fagni et al., 2000). Both DAG and $Ca^{2+}$ activate protein kinase C (PKC) (Hermans and Challiss, 2001). PKC then activates a cascade of second messenger enzymes and eventually leads to the phosphorylation of extracellular signal-regulated kinases (ERK)1/2 (Pawson, 1995), which is a central hub for protein translation: phospho-ERK1/2

can trigger translational activities (via Mnk1 and S6kinase) (Waskiewicz et al., 1997) as well as stimulate transcriptional factors (Elk1, CREB) (Davis, 1995). On the other hand, ERK phosphorylation is also tightly regulated by phosphatases such as PP2A (Haccard et al., 1990; Gause et al., 1993).

The importance of this ERK regulated translational and transcriptional control has been investigated by several experiments using inhibitors of MEK, the only kinase that activates ERK. Atkins et al. showed ERK activation is required for the expression of long-term memory induced by a fear-conditioning paradigm in rats, while inhibition of ERK activation led to a blockade of memory formation upon testing with either the cued or the contextual conditioning paradigms (Atkins et al., 1998). Blum et al found that intra-hippocampal infusion of MEK inhibitor blocked acquisition of spatial memory in rats (Blum et al., 1999). Waltz et al reported inhibition of ERK activation in the entorhinal cortex or in hippocampal area CA1 of rats impaired retention of avoidance in the inhibitory avoidance training paradigm (Walz et al., 1999; Walz et al., 2000).

Besides the ERK pathway, another parallel signal pathway controlling stimulation-dependent translation is the PI3K-AKT-mTOR pathway. In synapses, mGluR stimulation also activates PI3K and components of the mTOR signaling cascade in turn mediate synaptic plasticity via regulation of local protein synthesis. New evidence revealed that mTOR activation also suppresses PP2A activity and keeps FMRP in its phosphorylated state(Narayanan et al., 2007). In my current research, I will focus on the MAPK pathway.

Based on previous research, the rapid initiation of protein translation in response to synaptic activation is impaired in KO mice (Weiler et al., 1997; Weiler et al., 2004) . In KO animals, some kinases (PI3K, PKC) and/or phosphatases (PP2A) may be dysregulated because of the loss of FMRP, which breaks the dynamic balance of ERK phosphorylation and leads to the deficit in protein translation. We demonstrated that this translation defect can be traced back to the kinetics of ERK phosphorylation because of the pivotal role ERK is playing in protein translation (Kim et al., 2008).

Because lymphocytes and neurons all share these highly coordinated signaling pathways from mGluR to ERK phosphorylation, we hypothesize the dysregulated pathways seen in FXS neurons could also affect lymphocytes. In addition, lymphocytes might even mirror the change in neurons when the subjects are under pharmaceutical treatment targeting mGluR activity.

The precise control of ERK phosphorylation is a dynamic equilibrium between kinases activated by multiple converging pathways and phosphatases. Phosphatases act as negative feedback mediators to keep ERK from persistent activation. In the ERK activation assay, we use a potent PKC activator PMA to override kinases, and induce maximum ERK phosphorylation. We hypothesize that the difference between the rate of activation may be caused by phosphatases. This study will focus on a major phosphatase in mammalian cells: PP2A.

PP2A is a ubiquitously expressed protein serine/threonine phosphatase. It accounts for 70% of the total serine/threonine phosphatase activity in mammalian

cells(Liu et al., 2005). In several studies, PP2A has been confirmed to be a negative regulator of ERK signaling: PP2A can dephosphorylate and inactivate ERK *in vitro* (Alessi et al., 1995), while treatment with the PP2A-selective inhibitor okadaic acid causes activation of ERK *in vivo* (Sonoda et al., 1997).

PP2A is comprised of a core enzyme, which is made up of catalytic subunit C and scaffold subunit A, and a regulatory subunit B (Cho and Xu, 2007). The activity of PP2A is determined by the holoenzyme composition the core enzyme (PP2A A/C) and a wide array of regulatory B subunits (Xu et al., 2006). PP2A activity is also regulated by post-translational modifications. Phosphorylation of subunit C at tyrosine-307 inhibits the recruitment of the regulatory B subunits to the core enzyme and leads to inhibition of PP2A phosphatase activity (Chen et al., 1992). Mao et al have proposed that stimulation of mGluR5 induces Src-dependent tyrosine phosphorylation of PP2A at Tyr307, transiently inactivating PP2A, permitting short-lived increased ERK phosphorylation (Mao et al., 2005).

Dysregulation of PP2A might lead to potentially debilitating diseases such as cancer and Alzheimer's disease (Liu et al., 2005; Wong et al., 2010). In FXS research, Castets et al showed that the catalytic subunit (PP2Ac) has a putative G-quartet in its 5'UTR, making it a possible candidate for an FMRP cargo (Castets et al., 2005). Narayanan et al showed mGluR1 triggers a short activation of PP2A, giving a brief period of FMRP dephosphorylation, and enabling protein translation (Narayanan et al., 2007).

**ERK biomarker assay in clinical trials**

To test the ERK biomarker assay in clinical trial environment, we conducted two clinical trials on FXS subjects, collaborating with Dr. Elizabeth Berry-Kravis and Dr. Craig Erickson respectively.

**Clinical trial using Lithium**

For centuries, lithium has been commonly used as a mood stabilizing drug, primarily in the treatment of bipolar disorder and schizophrenia, to reduce manic behavior and depression (Baldessarini et al., 2006). Despite its proven therapeutic efficacy, the precise molecular mechanism of lithium as a mood-stabilizing agent is poorly understood. Currently, it is commonly accepted that lithium inhibits inositol trisphosphate synthesis and recycling, utilized by mGluR and other receptors to activate dendritic translation (Allison and Stewart, 1971; Kofman and Belmaker, 1993). Besides, lithium has been proven to inhibit several cell kinases, most notably GSK3β (Klein and Melton, 1996). It deactivates GSK3β by competing with Magnesium (Ryves and Harwood, 2001; Ryves et al., 2002). GSK3 is a molecule that in its nonphosphorylated state supports apoptosis (Chin et al., 2005), and has been suggested to be a negative regulator of ERK (Wang et al., 2005).

Lithium might theoretically correct excessive dendritic translation in FXS by acting as an inhibitory agent on signaling pathways that regulate translation. In Drosophila, lithium has been shown to improve FX-associated defects in naive courtship behavior, immediate recall and short-term memory in *dfxr* mutant flies (McBride et al.,

2005) and to reduce audiogenic seizures in the KO mouse model (Min et al., 2009). A recent publication by Choi et al suggested that chronic lithium treatment restored normal mGluR-LTD in KO mice (Choi et al., 2011). These preclinical findings suggested that lithium might provide therapeutic benefits for behavior and/or cognition in humans with FXS.

In this pilot study we systematically explored the effects of short-term (2 month) treatment with lithium on a broad range of phenotypes including behavior, cognition, and biophysical measures in a small cohort of FXS subjects. ERK is used as a biomarker to examine if this treatment would elicit improvements in biochemical performance that might correlate with improvements on behavioral scales.

**ERK assay in a clinical trial using Riluzole**

Riluzole is United States Food and Drug Administration (FDA)-approved (dosing 100 mg/day) for the treatment of amyotrophic lateral sclerosis (ALS) in adults. While the exact mechanism of Riluzole is unknown, the drug is thought to have neuroprotective effects in ALS, potentially related to the attenuation of glutamate induced excitotoxicity (Obrenovitch, 1998).

Riluzole is hypothesized to inhibit release of glutamate through inhibition both of voltage-dependent sodium channels and of P/Q-type calcium channels (Martin et al., 1993), and enhance glutamate reuptake (Frizzo et al., 2004; Fumagalli et al., 2008). Preclinical study has also pointed to an impact on GABA neurotransmission, including potentiation of post-synaptic GABA(A) receptor activity (Jahn et al., 2008) and blockage

of GABA uptake (Mantz et al., 1994). Riluzole also has anti-epileptic properties potentially related more to anti-glutamatergic than GABAergic effects (Kim et al., 2007).

Riluzole has been studied in several areas of psychopathology, including depression (Zarate et al., 2004), bipolar disorder (Zarate et al., 2005), obsessive-compulsive disorder (Coric et al., 2005; Grant et al., 2007) and trichotillomania (Coric et al., 2007). Major potential adverse effects of Riluzole use include elevated liver function tests (up to 50% of treated patients), neutropenia, reductions in hemoglobin, hematocrit, and erythrocyte counts, and interstitial lung disease (Sanofi-Aventis, 2009).

Given the potential mechanisms of action of Riluzole, combined with early positive reports on targeting treatment resistant OCD, we conducted the first study of Riluzole in FXS targeting interfering repetitive, compulsive behavior. ERK assays were performed before and after Riluzole treatment to provide quantitative assessment.

**FXS and dysregulated immune profiles**

Fragile X syndrome was first described in 1993. In decades of research to unveil the etiology and generate possible treatments, very little research focused on the immune system. It has been reported that in early childhood, Fragile X syndrome patients have on increased frequency of infections, especially otitis and sinusitis infections (Hagerman and Hagerman, 2002). In adulthood, more than 30% of patients have persistent gastrointestinal (GI) symptoms (Utari et al., 2010). However, whether Fragile X syndrome patients have more chances to get autoimmune disease or cancer has not yet been reported.

In 2007, Nishimura et al. first reported a genome-wide gene expression profile study of Fragile X syndrome patients (Nishimura et al., 2007). They found 120 genes that were differentially expressed. These genes include cellular signal transduction proteins and cell membrane receptors; however, the functional connections between these differentially expressed genes were not intensively studied.

To extract more information from the Nishimura's data, I used 2 successful statistical algorithms to re-analyze the raw data shared in Gene Expression Omnibus (GEO) database, at the level of gene sets. Gene sets are prior defined sets of genes based on biological knowledge, they group genes based on their biological process, molecular function and cellular component (Ashburner et al., 2000). Results showed 4 gene sets that are differentially expressed in FXS patients: structural constituents of the cytoskeletons, cell-cell adhesion inflammatory responses, inflammatory responses and humoral defense mechanisms. Interestingly, a large portion of the genes in those gene sets encode cytokines.

Cytokines are small, secreted proteins produced mostly by immune cells. Cytokines were originally characterized as immune modulators, however, more recent research suggested that cytokines can also regulate a diverse array of functions in some nonimmune tissues, including the central nervous system (CNS). Cytokines can modulate brain function, affecting cognitive and emotional processing, and exert assorted effects on neuronal tissue, such as the modulation of systemic and CNS

responses to infection, injury, and inflammation. The cytokine milieu has been shown to directly affect neural tissue function and development.

There has been very little research focused on cytokine profiles in FXS patients, however, there has been some cytokine profiling in Autism spectrum disorders (ASD). ASD are part of a broad spectrum of neurodevelopmental disorders, and are commonly seen in FXS patients (Hagerman and Hagerman, 2002). Ashwood et al found elevated IL-1β, IL-6, IL-8, IL-12, p40 and GM-CSF in ASD patients' plasma samples (Ashwood et al., 2010). Li et al found that TNF-α, IL-6 and GM-CSF, IFN-γ and chemokine IL-8 were significantly increased in the brains of ASD patients compared with controls (Li et al., 2009). In another study, increased cytokine IL-1β, IL-6, TNFα, TNFRI, TNFRII and MCP-1 level was found in CSF and brain tissues from ASD patients (Vargas et al., 2005).

There are several limitations in the cited reports: most research had to use frozen plasma or brain tissue samples, and the freeze-thaw cycle could cause small molecules like cytokines to denature/degrade. Moreover, those early reports were limited by the varieties of the cytokine multiplex beads they could use. Most of the reports mentioned above only chose 10-15 cytokines of interest, while valuable information from uninvestigated cytokines remains unknown. Besides, most research only investigated how basal cytokine profiles were different between groups; no further tests were done to reveal if cytokine production in response to infection is also different or not.

Given the results from microarray data analysis, there are reasons to believe the immune system in FXS patients may be dysregulated. We hypothesize, cytokines, as key mediators of cell-cell communication in the immune system, may be dysregulated in FXS patients as well.

Another closely related question is how well FXS patients respond to infections. Lipopolysaccharide (LPS) is a major constituent of Gram-negative bacterial cell walls. LPS has been documented to induce the expression of a large array of cytokines when it is administrated to peripheral blood mononuclear cells (PBMCs) (Raetz and Whitfield, 2002). Clinically, LPS challenge is frequently used to evaluate the functionality of the immune system (Virtala et al., 2011). In FXS, dysfunction in the response of PBMCs could result in long term alterations in immune system and could lead to adverse neuro-immune interactions.

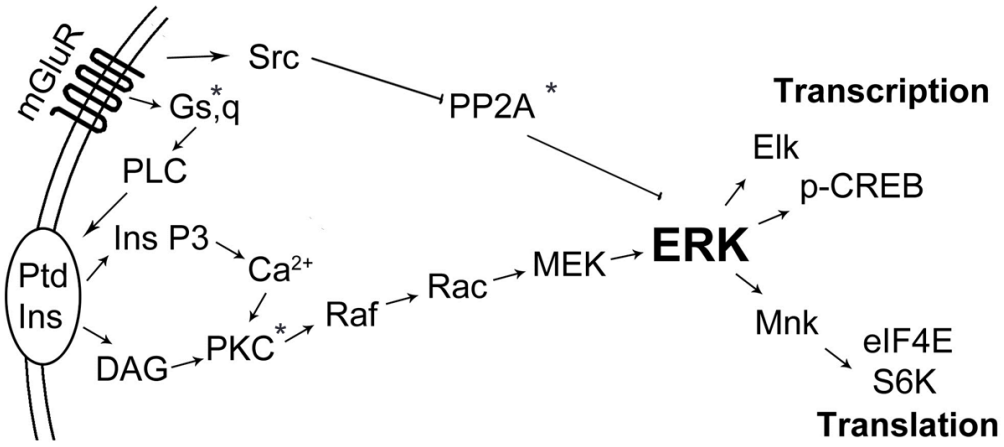
In this study, we measured the cytokine profiles in plasma samples from both FXS and typically developing controls, using a panel of antibodies against 36 different cytokines and chemokines. In addition, we isolated PBMCs from both FXS patients and typically developing controls, challenged these cell cultures *in vitro* with LPS, and examined the cytokine production in response to this prototypical endotoxin.

This research provides a general knowledge about the immune status of FXS patients. The results will help us to pinpoint which cytokine pathways are dysregulated and guide future research.

**Figures**

**Figure 1.1 Mechanism of translational activation and ERK**

Second-messenger cascade leading from neuronal Group I metabotropic glutamate receptors (mGluR), activation of Gs and Gq proteins, activation of phospholipase C that cleaves membrane phosphatidyl inositol (Ptd Ins) into inositol triphosphate (that releases $Ca^{2+}$ from intracellular stores) and diacylglycerol (DAG), both of which activate protein kinase C. Recruitment of Raf, Rac and MEK (Map/Erk kinase) results in ERK activation, resulting in both transcription and translation effects in the cell. The proteins with * superscript are putative FMRP cargoes.



## CHAPTER 2

## ERK ACTIVATION IS DYSREGULATED IN LYMPHOCYTES[1]

### Introduction

Fragile X Syndrome is a genetic disorder in which a single protein, FMRP, is not produced. As a result, localized translation of mRNAs bound by FMRP is not properly regulated (Weiler et al., 2004), leading to a wide range of cognitive and behavioral symptoms in FXS patients. Because it is difficult to standardize measurements of these symptoms, it is problematic to assess the effects of pharmacological treatment.

Among the mRNAs whose translation is modulated by transport and stimulation-evoked release by FMRP are enzymes (PI3K, PP2A, VHR, (Miyashiro et al., 2003)) that participate in signaling cascades leading to phosphorylation of the extracellular-signal regulated kinase (ERK). ERK is a nodal point for the convergence of at least 3 cell signaling cascades. Because of its central position, it has been used for detecting altered cellular activation states during drug treatments in tumor patients. Hedley and associates (Chow et al., 2005) pioneered the method of stimulating whole blood cell suspensions with phorbol ester for 10 minutes, followed by fixation, lysis of

[1] Data presented in this chapter were originally published in 3 peer-reviewed articles:

Early-phase ERK activation as a biomarker for metabolic status in fragile X syndrome.Weng N, Weiler IJ, Sumis A, Berry-Kravis E, Greenough WT.Am J Med Genet B Neuropsychiatr Genet. 2008 Oct 5;147B(7):1253-7.

Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. Berry-Kravis E, Sumis A, Hervey C, Nelson M, Porges SW, Weng N, Weiler IJ, Greenough WT.J Dev Behav Pediatr. 2008 Aug;29(4):293-302

Open-label Riluzole in fragile X syndrome. Erickson CA, Weng N, Weiler IJ, Greenough WT, Stigler KA, Wink LK, McDougle CJ. Brain Res. 2011 Mar 22;1380:264-70. Epub 2010 Nov 5

erythrocytes, and testing ERK activation of leukocytes in a flow cytometer. In otherwise healthy individuals, peripheral blood cells may mirror subtle differences in metabolic cascades associated with mood disorders (Li et al., 2007).

Because of our finding that rapid initiation of synaptic protein translation was defective in *fmr-1* KO mice (Weiler et al., 2004), we examined rapid phosphorylation of ERK in both neuronal (synaptic) samples and intact thymocytes of these mice, and found that KO mice were defective in early-onset ERK activation. Now, we have adapted flow cytometry methodology to timed sampling of isolated leucocytes from patient whole blood, measuring phospho-ERK at a series of time points immediately after phorbol ester stimulation. Using this method to determine the kinetics of early reactivity of leucocytes, we have observed that subjects with FXS show a slower onset of ERK activation than do unaffected individuals, paralleling results in thymocytes and purified synapses in *fmr-1* KO mice. We also demonstrated that dysregulated phosphatase activity leads to this delayed ERK phosphorylation. In PMA activated lymphocytes from normal donors, we observed a short period of PP2A inactivation but not in FXS patients.

Lithium and Riluzole are FDA approved drugs for the treatment of bipolar disorder and amyotrophic lateral sclerosis (ALS) respectively. They have been studied in several area of psychopathology, and could be beneficial for FXS subjects. In these two clinical trials, an ERK biomarker assay was used to examine whether these two treatments would elicit improvements in biochemical performance that might correlate with improvements on behavioral scales.

**Methods and materials**

1. Synaptoneurosomes stimulation.

Synaptoneurosomes were prepared from the cortices of single WT or fmr-1 KO mice (P13-P15) of the strain FVB.129P2-FMR1$^{tm1Cgr}$. Briefly, mice were quickly decapitated, brains were removed and dissected, and cortices were homogenized in a glass homogenizer in homogenizing buffer (50 mM HEPES pH 7.5, 125 mM NaCl, 100 mM sucrose, 2 mM potassium acetate), filtered through a series of nylon mesh filters (149, 62, 30 microns; Small Parts Inc.) and finally a 10 micron polypropylene Filter (Gelman). The final filtrate was spun briefly (400 g, 1 min); final volume was about 1 ml. Before stimulation, this suspension was incubated, stirring with 1 µM tetrodotoxin, on ice for 5 min then at RT for another 5 min. Synaptoneurosomes were then stimulated with 1 µM (S)-3,5-dihydroxyphenylglycine (DHPG) and samples were removed at 1, 2, 5, 10 minutes intervals. Reactions proceed at room temperature.

2. Thymocytes stimulation.

For mouse thymocytes, WT or fmr-1 KO mice were used. The thymus was removed from P10-P15 mice, and thymocytes were liberated from the capsule by gentle raking with a square of stainless-steel mesh. Cells were washed in PBS, resuspended at $10^6$ cells per ml in PBS, and stimulated by addition of phorbol myristate acetate (PMA, final concentration 40 nM). Cell aliquots were lysed in lysis buffer (50 mM Tris pH 8, 50 mM NaCl, 1% NP40, 2.5 mM sodium pyrophosphate, 1X Sigma protease inhibitor, 0.1 mM sodium vanadate) and applied to SDS-PAGE-electrophoresis, blotted to

nitrocellulose, and stained with rabbit monoclonal antibody specific to ERK phosphorylated at Thr202/Tyr204 (Cell Signaling Technology). HRP-labeled secondary anti-rabbit antibody was detected by enhanced chemiluminescence (Sigma or Pierce). To quantify protein levels in each lane, total protein was stained with Sypro Ruby. Chemiluminescence was scanned in an Alpha-Innotech imager, and relative optical densities were determined, normalized to total protein loaded.

3. Human Blood cells.

For normal control blood from anonymous donors at the local Community Blood Services, leucocyte-depletion filters, used to remove leucocytes from donated blood, were back-flushed with 20 ml HBSS containing 0.02% EDTA, and assayed within 24 hrs. Of this cell suspension, more concentrated than whole blood, 3 ml was layered on Ficoll Histopaque.

Male FXS participants and comparator control males were recruited from the Fragile X Clinic at Rush University Medical Center (RUMC) in Chicago and Fragile X Research and Treatment Center at Indiana University-Purdue University Indianapolis hospital (IUPUI) in Indianapolis. All control and FXS subjects or their legal guardians signed informed consent and assent as appropriate for study participation. The study was approved by the RUMC Institutional Review Board. All FXS subjects were positive for a fully methylated expansion mutation in *Fmr1* by DNA analysis. About 10 ml blood was drawn into EDTA-containing tubes, chilled, mailed by overnight delivery from RUMC/IUPUI to University of Illinois-Urbana, and used within 24 to 36 hours. All

samples were coded by number at RUMC/IUPUI and investigators assaying samples at University of Illinois were blinded to FXS status until assays were completed and data had been sent to RUMC, at which time mutation status and age only were released for subject grouping and age comparisons.

4. Flow cytometry.

About 3 ml of whole blood (or of concentrated leucocyte filter eluate) was layered onto 3 ml Histopaque in a 7 ml centrifuge tube, and centrifuged for 35 min at 400 g. The lymphocyte-containing layer was removed and transferred into RPMI-1640 for washing. After a second wash in RPMI-1640, cells were resuspended at $10^6$ cells per ml in RPMI-1640 and rested for 30 minutes.

Cells were stimulated by addition of PMA (as for thymocytes, above) to activate PKC and stimulate ERK phosphorylation; sample aliquots were removed at short intervals (1', 2', 3, 4', 5', 6', 10', 20') and fixed in 2% paraformaldehyde for 10 minutes.

Cells were permeabilized by addition of cold methanol for 30 min, followed by two washes in PBS with 2% FBS (FACS wash buffer). Fixed, permeabilized cells were stained by addition of Alexafluor488-labeled monoclonal antibody to phospho-ERK (Becton Dickinson) in the dark for 30 minutes, followed by two washes in FACS wash buffer. Resuspended cells were analyzed in a Coulter XL3 flow cytometer. Brightness of a subgroup of leucocytes, defined by size and irregularity was traced through the series of time points. In a separate experiment, the lymphocytes population was confirmed by staining with anti-CD3 (T cell receptor) antibody. The increase in brightness, resulting

from increasing amounts of phosphorylated ERK, was mapped on a curve and a value for time to half-maximum phosphorylation was obtained for each blood sample.

5. Cell ELISA.

We also developed an alternative method to make the ERK assay compatible with High-Content Screening environment.

To remove macrophages and granulocytes from the leukocyte population, we layered Histopaque of 2 densities (1.077 and 1.119) and centrifuged the blood cells through both. However, only about 30% of the granulocytes were removed by this method.

In another approach, we incubated the leukocytes on petri dishes in modified LGM-3 culture medium (LONZA) at 37C° for 2 h, during this time the macrophages and granulocytes adhere and the lymphocytes can be decanted off. With this method, we successfully removed about 90% of the granulocytes, with 30-40% loss of lymphocytes.

Purified lymphocytes were then concentrated, plated on to a 96-well plate and cultured in RPMI-1640 medium for about 4 hours. After stimulation, cells were then fixed by addiotion of 100µl 2% warm paraformaldehyde solution at pre-defined time points, the plate was then centrifuged at 800rpm for 10 minutes in a horizontal plate centrifuge. After centrifuging, the supernatants were removed and the plate was air-dried thoroughly. Cells were permeabilized by 2% Triton-X in PBS solution for 10

minutes. After washing, intracellular pERK signal was detected by Alexafluor488-labeled monoclonal pERK antibody (Becton Dickinson) using regular staining technique.

6. ERK-Directed phosphatase assay.

Timed samples of lymphocytes were lysed in a lysis buffer without phosphatase inhibitors. 1μg of total lysate was then incubated with 6ng recombinant phosphorylated $His_6$-pERK2 (Biomol, Plymouth Meeting, PA) dissolved in phosphatase assay buffer (10 mM $MgCl_2$, 10 mM HEPES, pH 7.5, and 10 μM of the MEK inhibitor U0126 to prevent ERK phosphorylation). After 10 minutes incubation at room temperature, the reactions were stopped by the addition of an equal volume of stop buffer (8 M Urea, 10 mM imidazole pH 8.6) and transferred to a His-Grab $Ni^{2+}$-coated ELISA plate (Pierce). After 90 minutes gentle rocking at 4C°, $His_6$-pERK was captured by the $Ni^{2+}$-coated ELISA plate. The plate was washed with stop buffer twice and wash buffer (300mM NaCl, 25mM Tris, pH 7.5) twice, and adherent p-ERK was detected by standard ELISA procedures. The value in each well represents $His_6$-pERK level after reaction, when compared with negative control which contains only lysis buffer and His-pERK, the loss of phosphorylated form of His-ERK corresponds to the amount or the activity of the phosphatase in the lysate. For positive control, His-pERK sample was incubated with 200 unit λ-phosphatase.

The amount of total ERK was confirmed by a following ELISA procedure to detect ERK. The plate was washed twice by stop buffer to remove pERK antibodies and twice with wash buffer. The adherent ERK was detected by standard ELISA using ERK antibody.

7. Flow cytometry with Dylight-633 pPP2A antibody.

Monoclonal p-PP2A antibody (Santa Cruz Biotech,CA) was raised against C-terminal amino acids 302-309 phosphorylated at Tyr307 of PP2Ac of human origin. We used a Dylight-633 NHS-Ester protein labeling kit from Thermo Scientific to label p-PP2A antibody directly, this Dylight-633 is compatible with our AlexaFluor488-conjugated antibody to p-ERK with minimal spectrum overlap.

The antibody stock was concentrated by Amicon Ultra-4 centifugal filter devices, in the meantime, the diluent was changed to borate buffer containing 1% glycerol. The pPP2A antibody was labeled according to the directions of the manufacturer, and purified on a size-exclusion column. This produces a high level of labeling (2-2.7 moles fluor per mole protein). At this degree of labeling, the working concentration of conjugated antibody is about 0.03 mg/ml when the cell:antibody ratio is $10^6$ cells to 10 µl antibody.

8. Lithium treatment.

In the Lithium clinical trial, subjects (age between 6 and 30 years) were recruited to the Rush University FXS Clinic. Informed written consent was obtained from either the subject or the parent prior to participation. The study was approved by the Institutional Review Board (IRB) at Rush University Medical Center.

Oral lithium carbonate was started after the baseline visit, initially at a dose of 300 mg TID for subjects weighing more than 50 kg and at a dose of 20 mg/kg/day

rounded to the nearest 150 mg increment and divided into a TID dosing schedule to a maximum of 300 mg TID for subjects less than 50 kg. Lithium levels were measured 2 weeks, 4 weeks, and 2 months, after starting treatment, and the dose was titrated upwards at 2 weeks and 4 weeks as appropriate (in 150 or 300 mg increments) based on the measured levels, to obtain a level as high within the 0.8 –1.2 mEq/L range as tolerated. If significant side effects occurred at a specific dose, the dose was reduced to the highest previous dose on which side effects had not been present. The dose was reduced (also in 150 or 300 mg increments) for levels above 1.2 even if there were no side effects. The goal was to achieve a steady level within the range of 0.8 to 1.2 mEq/L for the final four weeks of the 2 month treatment period.

At the baseline visit and 2 months after lithium treatment, all subjects had an assessment including a series of behavior tests and screening blood tests. A Clinical Global Impression - Severity (CGI-S) scale and an Aberrant Behavior Checklist – Community Edition (ABC-C) test was used to assess the severity of behavioral dysfunction. An ERK assay score was used to measure the responsiveness of blood lymphocytes.

9. Riluzole treatment.

A 6-week, prospective, open-label study design was chosen to gather pilot data on riluzole in adults with FXS. In this study, riluzole use primarily targeted the repetitive and compulsive behavior that frequently occurs in persons with FXS. Examples of such behavior include repetitive checking, questioning, touching, and picking.

Six adults with FXS aged 19-24 were recruited to the Indiana University. Written informed consent was obtained from each participant's legal guardian. The Indiana University and University of Illinois Institutional Review Boards (IRBs) approved the study.

Riluzole is commercially available in 50 mg tablets with a FDA-approved dosing of 50 mg twice daily for treatment of ALS. For each subject, the drug was started at 50 mg at bedtime for 1 week followed by a dose increase to 50 mg twice daily for the duration of the study, as tolerated.

At the baseline visit and the end of this 6-week riluzole treatment, a CGI-S score was completed by the PI, and the ERK score was assayed to provide biochemical assessment.

**Results**

1. Synaptoneurosomes from fmr-1 KO mice are defective in rapid phosphorylation of ERK, compared with WT mice.

Figure 2.1 is a Western blot of successive timed aliquots from a single preparation of cortical synaptoneurosomes from KO and WT mice, stimulated by the Group 1 mGluR agonist DHPG and stained for p-ERK. This mode of stimulation was selected because we had shown, in prior work, that mGluR stimulation of WT synaptoneurosomes elicits rapid initiation of protein translation, downstream of activation of ERK1/2. In WT synaptoneurosomes, phosphorylation of ERK 1/2 reaches a

maximum within 1 minute; but in a parallel preparation of KO synaptic particles, phosphorylation within the first 10 minutes is very slight.

Since basal levels of p-ERK are not lower in *fmr1*-KO mice (unpublished data), the FXS defect lies in the lack of rapid response. We therefore surmised that a timing defect might be detectible in other tissues, such as cells of the immune system, and turned first to a readily obtainable pure source of immune cells, the thymus.

2. Stimulated thymocytes respond to PKC activation more slowly in fmr-1 KO mice.

Thymus cells were suspended in PBS and stimulated with PMA (phorbol myristate acetate, 40 nM), to stimulate the PKC pathway directly and circumvent the need for cell-specific membrane receptors. Aliquots of the suspension were removed at short intervals and immediately lysed, blotted and stained for p-ERK. Figure 2.2 shows that intact thymocytes of WT mice respond rapidly, but those of KO mice respond with a delay of 2-5 minutes. Interestingly, KO and WT responses differ at the level of the PKC mediated response, therefore, in the absence of a contribution by mGluR5 receptors.

3. FXS patients peripheral blood lymphocytes show delayed ERK activation in response to PMA.

Based on the observation that the early-phase PKC-activated p-ERK pathway is defective in cells of the mouse immune system, we asked whether measuring the kinetic characteristics of the metabolic cascade in immune cells from human patients could serve as a diagnostic tool. To do this, we took advantage of methods devised to use the

basal activation state of the ERK pathway in peripheral blood cells as a marker for effects of pharmacological agents on tumor cells. To measure ERK1/2 activation state, leucocytes were purified over Hypaque (avoiding an erythrocyte lysis step), then stimulated with PMA. Cells were sampled at a series of short time intervals, fixed, permeabilized and stained with a fluorescence-tagged antibody to p-ERK. The intensity of staining was measured in a Coulter XL3 flow cytometer.

The distribution of blood leucocytes is illustrated in Figure 2.3. The lymphocytes population was confirmed by a separate experiment marking T lymphocytes by anti-CD3 (T cell receptor, a surface molecule found exclusively on T cells) antibody, illustrated in Figure 2.4 . The brightness of the p-ERK stained lymphocyte population is measured in successive samples, and plotted as shown against time. Figure 2.5 showed representative curves for a Fragile X patient and an unaffected control patient. Lymphocytes in both patients reach similar final levels of ERK phosphorylation after 20 minutes.

We measured times for ½ maximum ERK activation in control blood from 2 sources. Back-flushed leucocyte depletion filters from the Community Blood Services organization yielded a set of data for half-maximum activation averaging 3.5 min (Figure. 2.6). As FXS blood was not available through the Community Blood Service, a separate set of controls was obtained matched for age, gender and processing conditions with samples from subjects with FXS. Blood samples from this group of 13 control male subjects (age 24.8 ±7.1) averaged 4.5 minutes, with considerably more

variability than blood samples from filters. Blood samples from the corresponding cohort of 13 male subjects with FXS and a fragile X full mutation (age 22.7 ± 13.1, p=NS for age difference between FXS and control groups) sent by the same route had an ERK time for ½ maximum activation that averaged 5.5 minutes (p=0.06 relative to controls, Figure. 5). Within the group of subjects with FXS, 5 subjects were on no medications, 5 were on one medication, and 3 were on two medications, while no controls were on medications. Medications included antidepressants (N=5), stimulants (N=1), clonidine (N=1), antipsychotics (N=4). The 5 subjects not on medication had activation times that clustered toward the higher end of the range for FXS, suggesting that the prolonged activation time in the FXS cohort was not due to medication effect. The unmedicated group with FXS had an ERK ½ maximum activation time that averaged 5.95 minutes (p=0.017 relative to controls, Figure 2.6).

4. An alternative method to measure the speed of ERK activation.

One limitation of the flow cytometry method described above is that it is not suitable for processing a large amount of samples. We developed an alternative method to make the ERK assay compatible with High-Content Screening environment.

As illustrated in Figure 2.7, pre-incubation with LGM-3 media successfully removed about 90% of the granulocytes, and gave relatively pure lymphocytes for further processing. However, about 30-40% of the lymphocytes were lost during the incubation step. This method generated similar results comparing with the regular flow cytometry method, as showed in figure 2.8.

5. Dysregulated phosphatase activity mediates delayed pERK response.

PMA is a potent PKC activator. In our test condition, consistently, PMA induced maximum ERK phosphorylation. The difference between the kinetics of ERK phosphorylation may be a result of elevated phosphatase activity in FXS samples. To test our hypothesis, we measured ERK-directed phosphatase activity at several early time points. As illustrated in Figure 2.9, phosphatase activity in control blood lymphocytes started to decrease after 1 minute of PMA treatment, reached its lowest level at 5 minutes, and recovered at 10 minutes. In FXS samples, phosphatase activity increased, reached a plateau at 2 minutes, and kept steady till 10 minutes, showing a completely different pattern from the control samples. Note that at baseline, phosphatase activity is lower in FXS, which may explain slightly higher phosphorylated ERK level detected in FXS cells (Figure 2.2).

6. Temporary PP2A deactivation may account for phosphatase activity change.

The ERK-directed phosphatase activity assay did not reveal which phosphatase/ phosphatases may account for the activity change. Since PP2A is a major protein serine/threonine phosphatase in mammalian cells, and accounts for 70% of the total serine/threonine phosphatase activity (Liu et al., 2005), we decided to examine PP2A phosphorylation.

Fluorophore- coupled anti phosphor-Tyr307 PP2Ac is not commercially available, therefore, we labeled phosphor-Tyr307 PP2Ac antibody with DyLight -633, and then used flow cytometry to measure timed lymphocytes samples from controls and FXS

subjects. In 4 control samples, there was a short (about 1-2 minutes) but strong phosphorylation of PP2A (5-fold higher than baseline), while in the FXS subjects, none of the 6 samples showed PP2A phosphorylation significantly different from baseline. The result indicated PMA induced a transient PP2A phosphorylation (deactivation) only in normal controls.

Interestingly, the spike of PP2A phosphorylation happened at 4-6minutes after PMA stimulation, this time-line matches the result shown Figure 2.9, where in the controls, ERK-directed phosphatase activity reached the lowest point at around 5 minutes, suggesting PP2A could be a major player that accounts for the sudden change in phosphatase activity.

7. Lithium treatment.

ERK activation times, defined as the time in minutes for ERK phosphorylation to reach the half maximal level, were successfully obtained at baseline and two months for 11 subjects, and decreased for all 11 subjects after lithium treatment (Figure 2.11). The baseline mean activation rate of 4.872 minutes was reduced to 4.109 minutes after 2 months of lithium treatment ($p = 0.007$). The ABC-C scores of these patients were significantly improved too, the mean score of 60.6 at baseline was reduced to 42.1 at end point , with a P value of 0.005. When we plotted the ABC-C score with ERK score, there is a weak linear correlation between the behavior measurement and the biochemical measurement, the correlation coefficient $R^2$ was 0.5073.

8. Riluzole treatment.

CGI-I scores range from 1-7, a CGI-I score of 1 means significantly improved, while a CGI-I score of 7 means significantly worse. After 6-week riluzole treatment targeting compulsive behavior, most subjects had a CGI-I score of 4, suggesting that Riluzole use was not associated with significant clinical improvement. Out of the 6 participants, the only clinical responder was subject #1, with a CGI-I score better than 3. Note that this subject was on Lithium treatment when he participated the clinical trial.

On the other hand, the normalization of ERK and clinical improvement were disconnected. After 6-week riluzole treatment. Mean ERK activation time was reduced from 3.8±0.3 minutes at baseline to 2.99±0.3 minutes at endpoint ($p=0.007$) Out of 6 subjects, subject #1 was the one that had the lowest ERK score at baseline, he was also the one showed almost no change in ERK score (from 3.51 minutes to 3.18 minutes).

**Discussion**

Fragile X Syndrome is the leading inherited cause of mental retardation in humans; it is often accompanied by attentional deficit, hyperactivity disorder and autism-spectrum symptoms. Other frequently seen symptoms include cognitive impairment, seizure susceptibility, hyperarousal, sensory hypersensitivity, and heightened anxiety (Berry-Kravis et al., 2002; Hagerman, 2002; Hatton et al., 2002).

Numerous psychotropic medications are being used in clinical practice, in an attempt to treat behavior problems of individuals with FXS (Hagerman, 2002; Berry-

Kravis and Potanos, 2004; Valdovinos, 2006) and new classes of medications are in development to target underlying glutamatergic mechanisms misregulated in FXS due to absence of FMRP (Berry-Kravis et al., 2006). Both anecdotal and behavioral testing methods have been employed with variable success in an attempt to measure improvement or lack thereof. Thus, particularly for future treatments targeted at disease mechanisms in FXS, it would be helpful to develop an enzyme-based method, usable on peripheral blood cells, that would yield a quantifiable score and serve as a biomarker for treatment of individuals with FXS. Peripheral blood cells have previously been successfully used with flow cytometry to establish an equilibrium level of ERK activation in lymphocytes of cancer patients (Chow et al., 2005; Tong et al., 2006). Human peripheral blood mononuclear cells have been used to study signaling pathways before and after lithium treatment of patients suffering from bipolar disorder (Li et al., 2007).

A central event in cell signaling, the activation (by phosphorylation) of the extracellular receptor regulated kinase (ERK) is delayed in both mice and humans lacking fragile X mental retardation protein (FMRP), probably caused by imbalances in enzymatic signaling systems in the absence of FMRP due to dysregulated localized translation of some members of the mRNA subset it binds. The observed effect does not appear to be caused by medications in use by humans with FXS because (1) subjects with FXS who were not treated with medication, if anything, tended to have longer activation times, and (2) the effect in humans is consistent with that in mice, in which medication exposure is not an issue. There are too few subjects in this cohort to

address the question of whether specific medications may actually lower ERK activation rates in FXS, although it is interesting to note that four of the five lowest activation times measured in subjects with FXS were in lymphocytes from subjects treated with selective serotonin reuptake inhibitors (SSRIs) to reduce anxiety. There is no prior literature to predict potential effects of SSRIs on ERK activation.

Stimulation of group 1 metabotropic glutamate receptors activates phospholipase C, splitting membrane phosphatidyl inositol into inositol triphosphate (releasing intracellular $Ca^{2+}$ from cytoplasmic stores) and diacylglycerol, a specific activator of protein kinase C (PKC). PKC activation triggers a cascade leading to MEK phosphorylation of ERK (Figure. 1.1). In these experiments, we directly stimulated PKC, thus circumventing other receptor-triggered effects that modulate the p-ERK response. This segment of the signaling cascade is present also in immune cells and serves as an indicator for state of responsiveness. To our knowledge, this is the first technique using kinetic analysis of the early phases of ERK activation to establish metabolic differences between lymphocytes from FXS patients and unaffected subjects. Measurement of the early-phase ERK activation response is particularly well qualified to quantify an imbalance of enzymes in a coordinated pathway in suspected cases of FXS and possibly other related syndromes, and might serve as well as an indicator for changes in responsiveness, for example as a result of treatment by pharmacological agents. In the clinical trials using lithium, improved ERK response can be correlated with the improvement in behavior test, showed good promise as a biomarker for future clinical trials targeting pathways of translational activation involving ERK signaling in FXS.

In the Riluzole clinical trial, there is a clear disconnection between normalization of ERK and clinical improvement. Even with the small subject number, mean ERK activation times decreased significantly with the change being noted in each subject tested. Despite this uniform decrease, the drug showed clinical benefit in only 1 of 6 subjects. This incongruence could have several explanations. It is possible that the correction of enzymatic dysregulation in FXS that may be represented by normalization of ERK activation does not correlate with clinical improvement in short-term drug trials. The ERK effect could be relatively rapid with potential neuronal adaptation and clinically evident change in the human brain taking more time.

The age of FXS patients studied may also provide an explanation. Adult FXS subjects with a history of aberrant brain development for decades may not be susceptible to rapid clinical benefit from drug treatment that may work to correct underlying neurochemical deficits. There also may be a difference between the peripheral and central response to riluzole. Riluzole may reach serum levels that rapidly correct aberrant ERK activation in blood cells, but the drug may lack a similar central nervous system effect. Potential underdosing of riluzole in this trial could also contribute. Other open-label reports targeting depression in adults used up to 200 mg/day (Zarate et al., 2004; Zarate et al., 2005). We limited riluzole dosing to the FDA-approved limit of 100 mg/day due to safety concerns in a high risk population often lacking the ability to adequately communicate potential drug-associated adverse effects. Decreased ERK activation may also represent a general marker of biochemical restoration that may not equate specifically with the neurobiological changes required

to correct the human FXS phenotype. These explanations point to the need for future trials of potential targeted treatments in FXS to extend treatment duration beyond standard short-term drug trials, while also considering the need to assess drug response in youth with FXS regardless of clinical findings in adults.

The single treatment responder had the smallest improvement in ERK activation time. Interestingly, the treatment responder was on concomitant lithium which might have resulted in a previously improved baseline, and limiting a further decrease in ERK activation. This single case raises the possibility of that use of a synergistic drug combination might result in improved response.

**Figures**

**Figure 2.1 DHPG induced ERK activation is impaired in KO mouse synaptoneurosomes**

ERK1/2 is rapidly phosphorylated in WT, but not *fmr-1* KO synaptoneurosomes after stimulation of metabotropic glutamate receptors. Synaptoneurosomes from *fmr-1* KO mice (left) and WT (right) were stimulated by 1 nM DHPG and samples were taken at 1', 2', 5', and 10'. Control, unstimulated synaptoneurosomes were sampled at 0' and 10'. Lysates were separated on an 8% polyacrylamide gel and stained for phosphorylated ERK. (n=8)

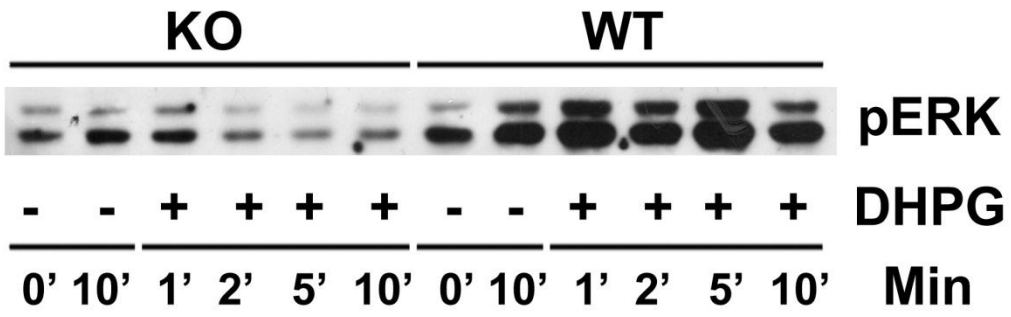
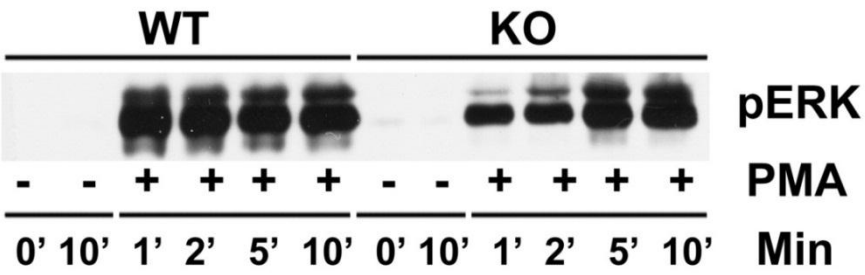


**Figure 2.2 KO mouse thymocytes show slower response upon PMA stimulation**

ERK1/2 is rapidly phosphorylated in WT, but not *fmr-1* thymocytes. Thymocyte suspensions ($10^6$ per ml) were prepared in parallel from P12 WT (left) and KO mice (right) and stimulated in a stirred suspension in PBS by 40 nM PMA; timed samples (1', 2', 5', 10') were lysed, separated on a gel, blotted and stained as in Figure2.1. Samples of unstimulated suspensions were lysed at t=0, 10'. (n=6)



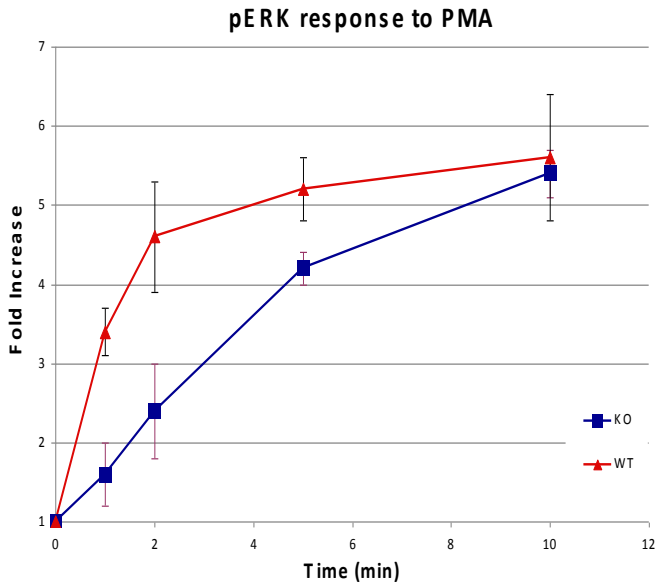



**Figure 2.3 Flow cytometry processing**

Stimulated, fixed, permeabilized blood leucocytes were stained with monoclonal antibody to p-EEK, conjugated with Alexafluor 488. The first box shows the analysis of leucocytes by forward scatter (FS) and side scatter (SS), separating the cells into roughly 3 populations, of which lymphocytes (circled, second box) are followed for changes in brightness (third box, showing number of cells in log intensity categories).

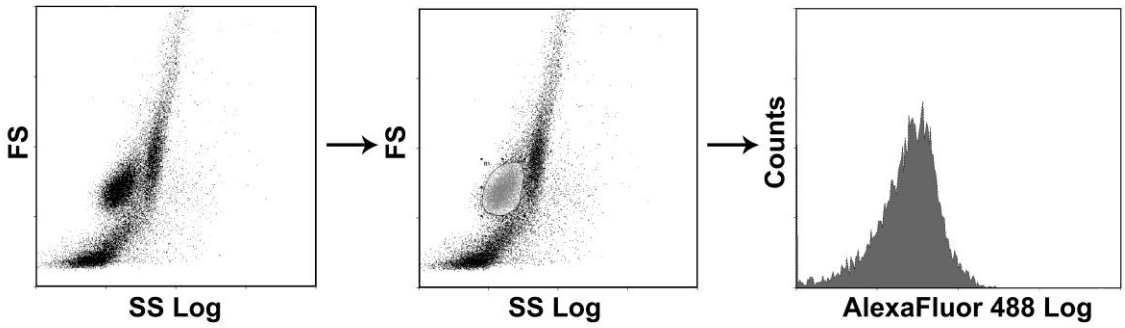


**Figure 2.4 Lymphocytes population was confirmed by CD3 (T-cell Receptor) staining**

CD3 (T lymphocytes specific surface marker, represented in green) stained part of the cell population in the middle, where the rest of the black particles in the population are B lymphocytes.

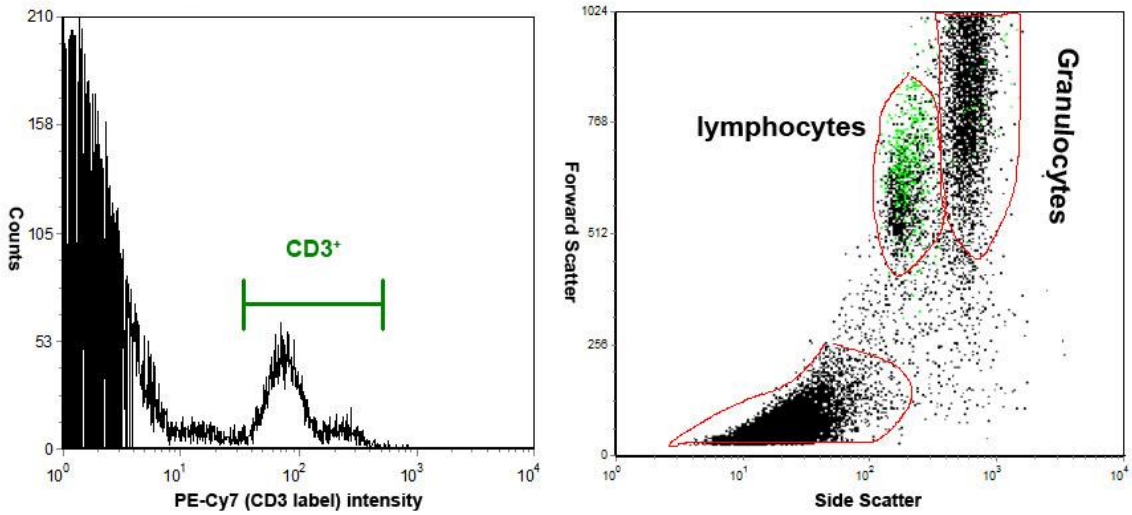


**Figure 2.5 ERK activation curve**

A. The fluorescence intensity profile of lymphocytes is measured at a series of time points. Time lines shown are t=0, 1', 2', 3', 4', 5', 6', 10', 20'.

B. From these measurements the fold increase in intensity is plotted; typical curves for a control subject (solid line) and a subject with FXS (dotted line). T=1/2 max, the time to reach half-maximum phosphorylation (brightest intensity) is taken as a measure of phosphorylation efficiency.

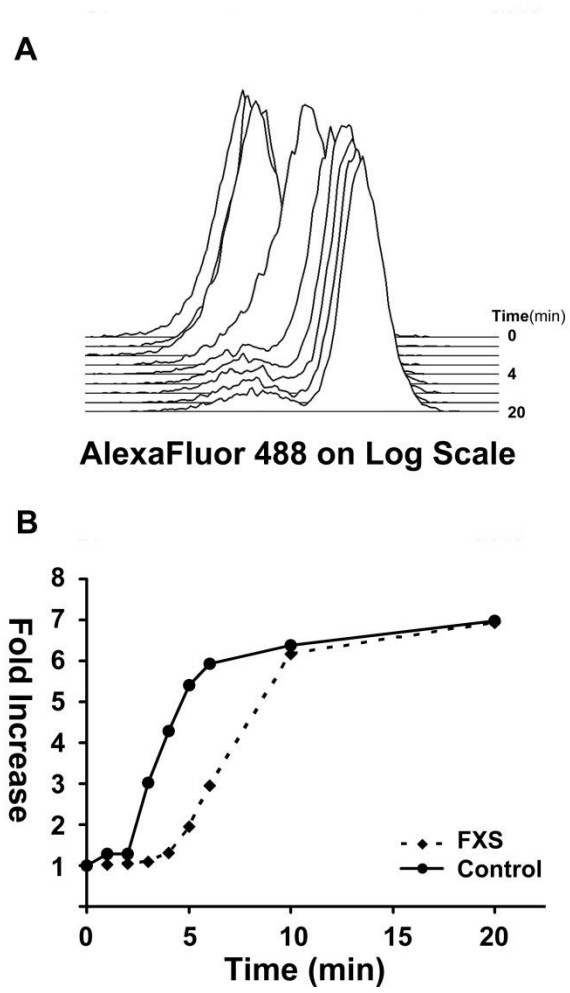


**Figure 2.6 FXS patients have higher T1/2 than controls**

Scattergram of t=1/2 max values for subjects with FXS (N=13), control subjects (N=13), and lymphocyte suspensions retrieved from blood bank leucocyte filters. Filled circles in the FXS column denote patients receiving one or more medications, see text. Not all points are visible on the graph due to overlap when two values are the same or very close. For each group, means are shown and error bars represent standard error of the mean (SEM).

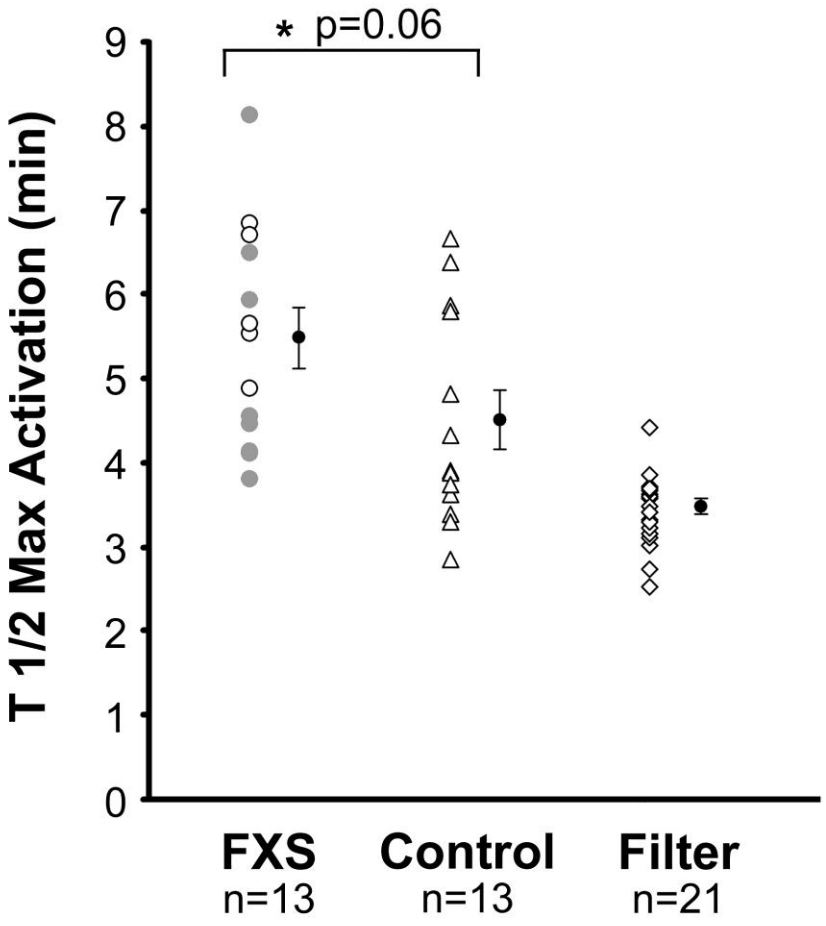
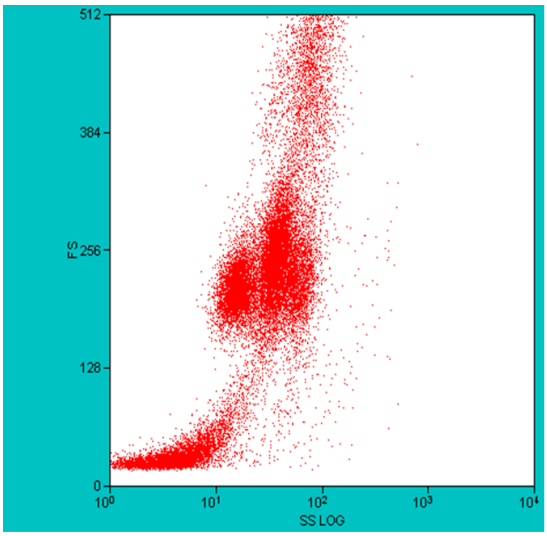
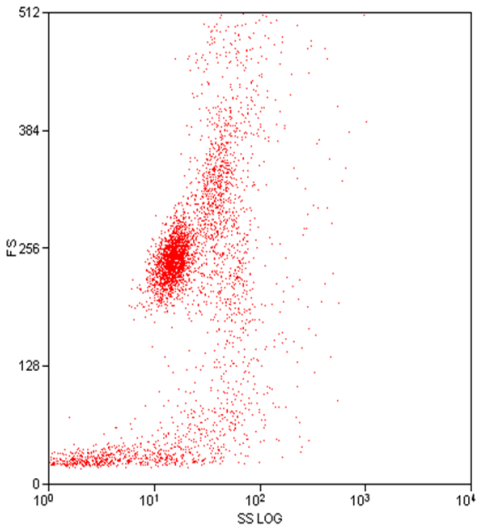


**Figure 2.7 Lymphocytes purification using LGM**

Pre-incubation of leukocytes in Lymphocytes growth medium (LGM) successfully removed 90% of the granulocytes (comparing the figure on the left), and generated a relatively pure lymphocyte population (showed in the figure on the right ) for further Cell-ELISA procedure. However, about 30% of the total lymphocytes were lost in the incubation step.




No incubation (Input)




2h incubation at 37C
In LGM w/1% FBS

**Figure 2.8 Lymphocytes recovered from LGM have similar ERK stimulation pattern**

Lymphocytes purified by LGM have very similar stimulation pattern comparing with lymphocytes recovered from Hipaque. The difference between the Cell-ELISA method (solid line) and the regular Flow-cytometry method (dash line) is very subtle.

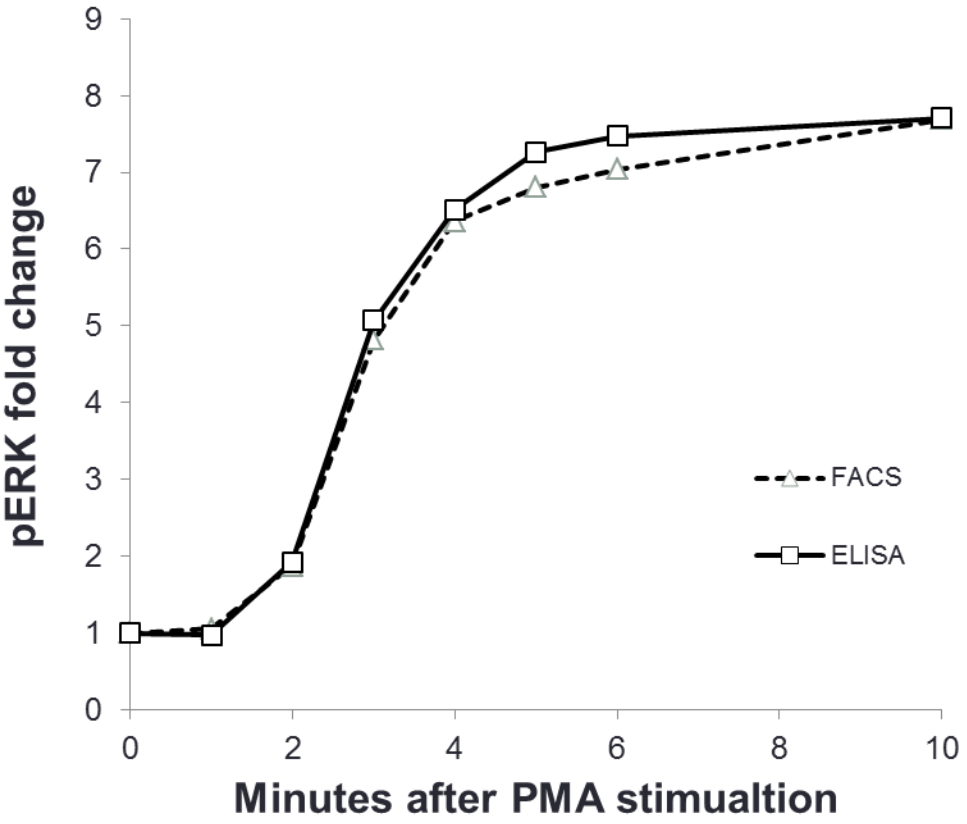


**Figure 2.9 ERK-directed phosphatase activity assay**

Human blood lymphocytes were stimulated by PMA, timed samples were lysed in a lysis buffer without phosphatase inhibitors but a MEK inhibitor U0126, then incubated with same amount of His6-tagged phospho-ERK. The amount of remaining pERK was quantified by ELISA. By measuring how much pERK was dephosphorylated, phosphatase activity at a specific time point could be determined. In Control samples, phosphatase activity was quickly decreased, to allow fast ERK phosphorylation, but this phenomenon was not seen in FXS subjects. (n=4)

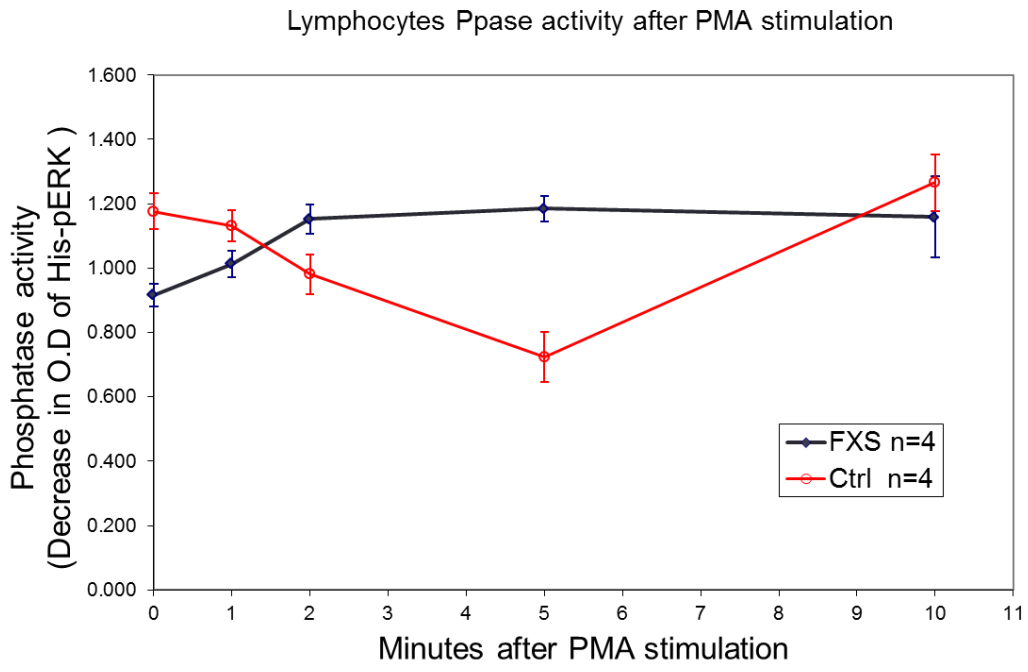


**Figure 2.10 PP2A is deactivated in controls but not in FXS after PMA stimulation**

Human blood lymphocytes were stimulated by PMA, timed samples were fixed. Tyr-307 phospho-PP2Ac was stained and measured by flow cytometry. In the 4 control samples, PP2A was transiently phosphorylated (deactived) in response to PMA; while in the 6 FXS samples, none of them showed any sign of PP2A phosphorylation.

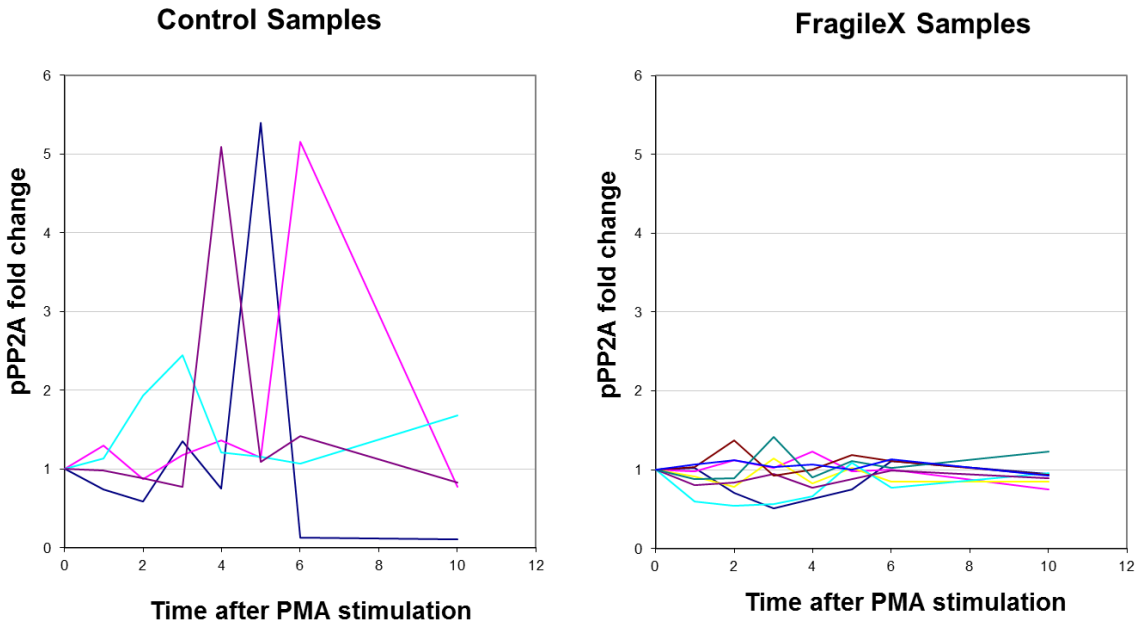


**Figure 2.11 2-month lithium treatment reduced ERK scores in FXS patients**

Change in ERK activation times from baseline to two months of lithium treatment in FXS study participants. Reduction of activation time (faster activation) represents normalization as FXS subjects show longer activation times than normal controls.

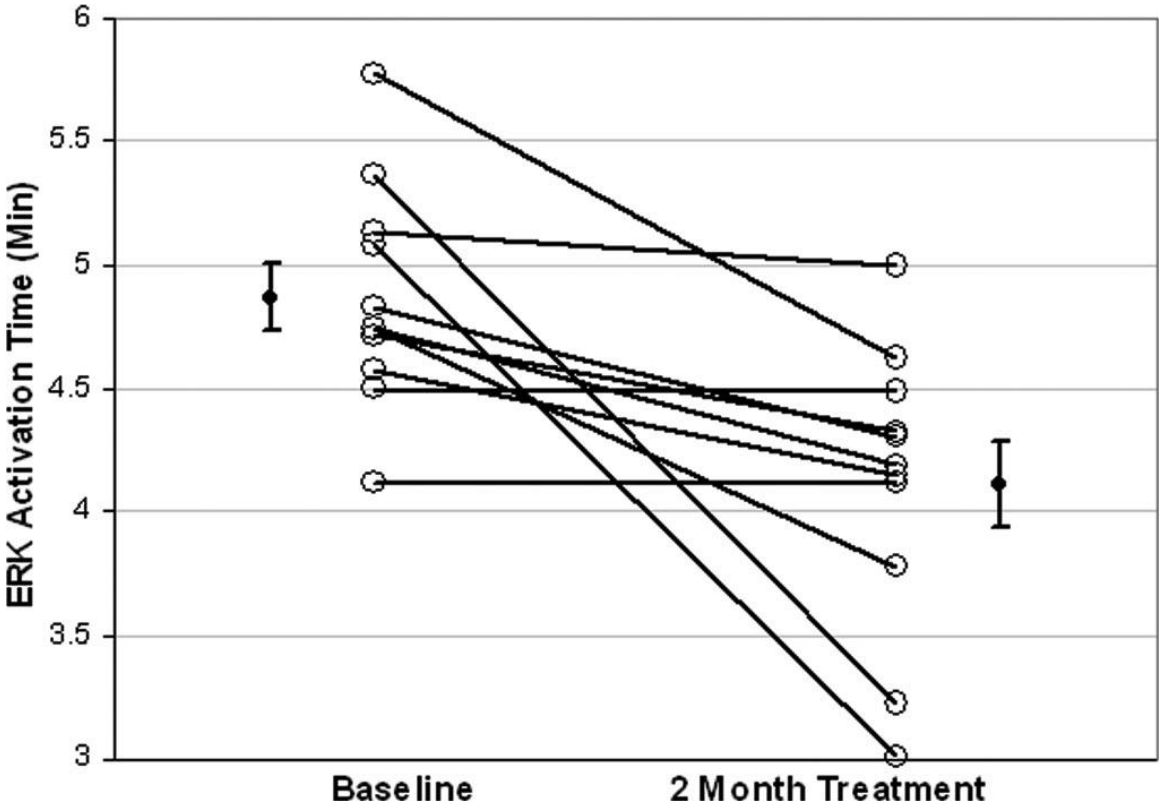


**Figure 2.12 Correlation between behavior and ERK biomarker score**

After 2-month Lithium treatment, the improvement in biochemical measurement (change in ERK score after exponential transfer) showed a week correlation with behavioral measurement (change in ABC-C after log transfer), the correlation coefficient $R^2$ was 0.5073.

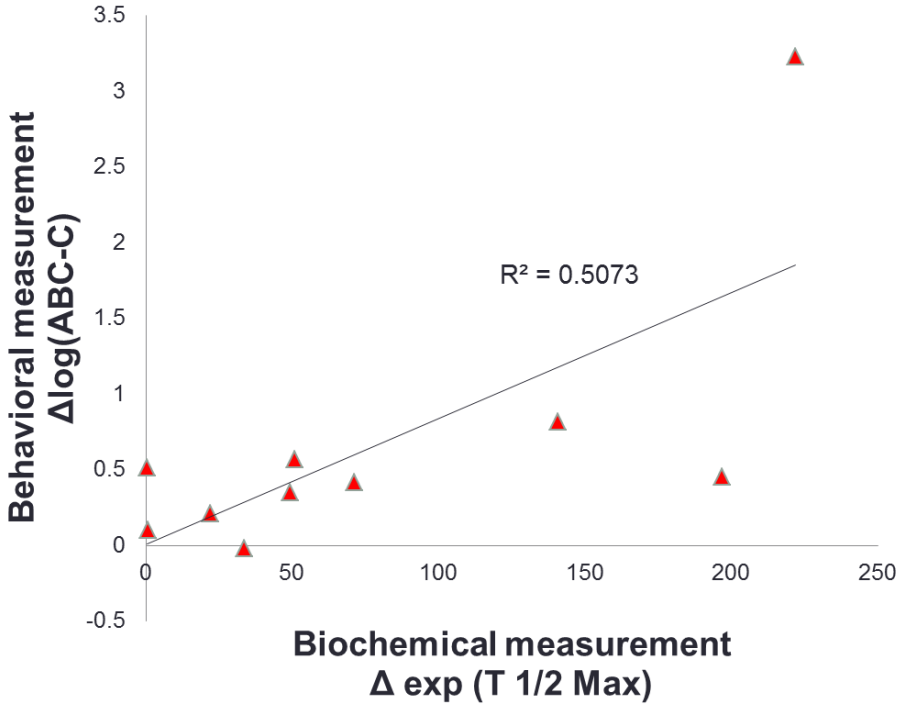


**Figure 2.13 Riluzole clinical trial**

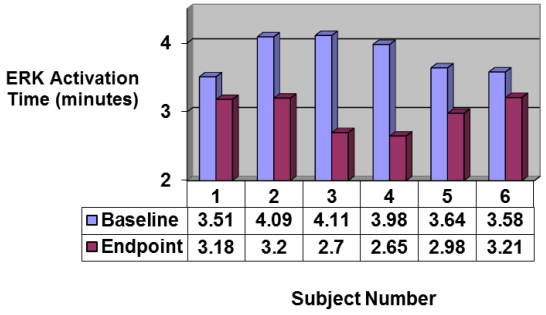
A: After 6-week riluzole treatment, out of the 6 subjects, the only responder is subject #1, with a CGI-I score higher than 3 (significantly improved). Note that this subject was on Lithium treatment when he participated the clinical trial.

B: ERK assay score before and after 6-week riluzole treatment. Mean ERK activation time was reduced from 3.8±0.3 min at baseline to 2.99±0.3 min at endpoint (p=0.007) Out of 6 subjects, subject #1 was the one that had the lowest ERK score at baseline, he was also the one showed almost no change in ERK score (from 3.51min to 3.18min)

A

| Patient | Age (years) | Concomitant Medications | CGI-I |
|---|---|---|---|
| 1 | 22 | Alprazolam, aripiprazole, fluvoxamine, lithium | 2 |
| 2 | 22 | Atomoxetine, quetiapine | 4 |
| 3 | 24 | Citalopram, methylphenidate ER | 4 |
| 4 | 24 | Escitalopram, methylphenidate ER | 4 |
| 5 | 19 | Aripiprazole | 3 |
| 6 | 24 | Citalopram, clonidine | 4 |

B



# CHAPTER 3

# DYSREGULATED IMMUNE PROFILES IN FXS PATIENTS

## Introduction

Fragile X syndrome was first described in the 1993. In decades of research to unveil the etiology and generate possible treatments, very little research focused on the immune system. It has been reported that in early childhood, Fragile X syndrome patients have increased frequency of infections, especially otitis and sinusitis infections (Hagerman and Hagerman, 2002). In adulthood, more than 30% of patients have persistent gastrointestinal (GI) symptoms (Utari et al., 2010). However, whether Fragile X syndrome patients are predisposed to autoimmune disease or cancer has not yet been reported.

At the cellular level, high levels of *Fmr1* transcripts are found in immune tissues like thymus and spleen (Pieretti et al., 1991; Hagerman and Hagerman, 2002). Since FMRP plays an important role in mRNA transportation and protein translation control, we hypothesize that immune cells from FXS patients may have different gene expression and protein profiles.

We tested our hypothesis at three different levels. We first analyzed a raw genome-wide microarray data from FXS lymphoblastoid cell lines to search for differentially expressed gene sets. The results showed that several immune gene sets are differentially expressed in FXS patients. This finding led us to look into cytokine

profiles. In addition, we challenged blood leukocytes with Lipopolysaccharides (LPS), and examined cytokines activation profiles.

**Methods and materials**

1. Microarray data analysis.

The microarray expression data from Nishimura's study was downloaded from Gene Expression Omnibus (GEO) public functional genomics database under accession number GSE7329. For that study, lymphocyte samples were collected from 15 normal donors and 8 Fragile X Syndrome patients. Total RNA from lymphocytes from each individual were labeled with Cy5 fluorescence. Reference targets were made by using pooled total RNA from the 15 normal individuals and labeled with Cy3 fluorescence.

For our analysis, we focused on probes with signal-to-noise ratio > 2.7 in both Cy3 and Cy5 in at least 14 of 15 controls. Normalization was performed using the LIMMA Package under R statistical software.

Gene set definitions were collected from 3 sources: Gene Ontology, KEGG and HumanPath database. A total of 600 gene set groups were used and covered about 10,000 gene probes on the array. Gene sets that are too small (contain less than 15 genes) or too large (contain more than 600 genes) were excluded from analysis. Gene Set Enrichment Analysis was performed using the GSA package under R statistical software. Fault Detection Rate (FDR) is controlled to be less than 0.25. SigPathway Analysis was performed by SigPathway software package on Bioconductor platform.

2. Study participants.

Male FXS participants (aged 16-28) and comparator control males were recruited by Dr. Berry-Kravis from Rush Medical Center, and by Dr. Craig Erickson from Indiana University-Purdue University Indianapolis. All control and FXS subjects or their legal guardians signed informed consent and assent as appropriate for study participation. The study was approved by the UIUC Institutional Review Board. All FXS subjects were positive for a fully methylated expansion mutation in *Fmr1* by DNA analysis. About 12 ml blood was drawn into EDTA-containing tubes, chilled, mailed by overnight delivery to University of Illinois-Urbana, and used within 20 to 24 hours.

3. Cytokine analysis.

For blood plasma cytokine analysis, about 3 ml blood was centrifuged at 300rpm for 20 minutes, the plasma on top of the pellet was collected (about 1.2 ml) for further testing.

For cytokine production measurement, leukocytes were isolated over Ficoll-histopaque gradients, and purified by 2 washes in RPMI-1640 culture medium. Cell number was counted, and $1x10^6$ cells were seeded in a 24-well culture plate for later analysis. Cells were first cultured in 1 ml complete medium (RPMI-1640 with 5% FBS) for 24 hours, then washed and switched to 1 ml RPMI without serum. At this time, treatment cells received 100 ng/ml LPS dissolved in saline while untreated cells received saline only. After 16 hours, culture medium samples were collected and centrifuged at

3000 rpm for 10 minutes to remove residual cells; the supernatants were used for further testing.

A cytokine analysis kit was purchased from R&D systems (ARY-005, Human Cytokine Array Kit, Panel A, Minneapolis, MN). The experiments were performed according to the manufacture's protocol. A total of 36 different cytokines/chemokines and acute phase proteins were assayed ( C5a, CD40 Ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α, sTREM-1). Fo the sake of clarity, only those cytokines/chemokines with levels significantly different between FXS and TD will be shown in the result figures.

4. Statistical analysis.

The relative amounts of each cytokine were standardized by the 3 groups of positive control dots on the membrane, to control for exposure difference between experiments. Student's t test was used for statistical comparison of FXS and Control samples. ANOVA was done for comparison of more than two groups.

**Results**

1. Microarray data analysis.

About 200 gene sets were analyzed and ordered by either P-value or Rank scores. Combining sigPathway and GSA results, we were able to identify 4 gene sets that

are differentially expressed (P < 0.05) between FXS and unaffected individuals: structural constituents of cytoskeleton, cell-cell adhesion inflammatory responses, inflammatory responses and humoral defense mechanisms.

| Pathway | GSEA p- value | GSA p-value | SigPathway Rank |
|---|---|---|---|
| structural constituents of cytoskeleton | 0 | 0.02 | 5 |
| cell-cell adhesion inflammatory responses | 0.01 | 0.01 | 6 |
| inflammatory responses | 0.01 | 0 | 10 |
| humoral defense mechanisms | 0.02 | 0.04 | 13 |

Figure 3.1. shows the genes in “inflammatory response”. A large portion of the genes encode cytokine/chemokines. Most of those genes were up-regulated in FXS.

2. Plasma cytokine profiles.

Out of 36 cytokines/chemokines, 3 cytokines (IL-1β, IL-27 and TNF-α) and one chemokine (IL-8) levels are significantly different in control plasma samples from FXS subjects. Three are present at higher levels in FXS plasma; one (IL-27) is lower (Figure 3.2)

3. Active Cytokine production.

To demonstrate basal cytokine production before LPS stimulation, we cultured PBMCs in serum-free medium, and measured secretion into the culture medium. As illustrated in figure 3.3, after culturing PBMCs for 16 hours in this conditioned medium,

3 cytokines and 2 chemokines : IL-1β, IL-1ra, IL-8, TNF-α and RANTES are significantly higher in the FXS group, while IL-27 is lower in FXS. Interestingly, 4 of these cytokines, IL-1β, IL-8, IL-27, TNF-α correspond to the plasma cytokine results showed above, two (IL-1ra and RANTES) can be detected only after culture.

3. LPS-induced cytokine release.

LPS potently triggered pro-inflammatory cytokine release. After 16 hours in LPS, PBMCs from FXS patients produced significantly higher levels of GM-CSF, IL-1β, IL-16 and TNF-α than TD controls. Two of the 4 cytokines (GM-CSF and IL16) are specific to the LPS response; and all are more strongly produced in FXS than control subjects.

**Discussion**

In early research, *in situ* hybridization studies showed that high levels of *Fmr1* transcripts are found in immune tissues like thymus and spleen (Pieretti et al., 1991; Hagerman and Hagerman, 2002), where T-lymphocytes and B-lymphocytes are produced. Since FMRP plays an important role in mRNA transportation and protein translation control, expression of genes related to immunity may be affected by the loss of FMRP. Our research is the first to categorize those differentially expressed genes, based on microarray analysis. A potential altered gene expression pattern in FXS patients led us to further investigate cytokines in samples from FXS patients.

1. Related studies.

Cytokine profiles in FXS has not yet been studied, however, cytokine profiles from ASD patients have been reported by several research groups. Although the etiology between ASD and FXS is different, about 30% of FXS patients also have ASD (Hagerman and Hagerman, 2002), and even more display autistic behaviors similar to ASD (Clifford et al., 2007; Bailey et al., 2008) . In ASD research, a clear pattern has emerged in several studies that shows altered levels of immune mediators are associated with increased impairment in behavior(Ashwood and Van de Water, 2004; Onore et al., 2009; Ashwood et al., 2010; Ashwood et al., 2010; Careaga et al., 2010; Chonchaiya et al., 2010; Enstrom et al., 2010). Significantly higher cytokines in children with ASD are linked to behavior impairments in ASD. Our finding in FXS is consistent with those observations in ASD patients, suggesting a common pattern between ASD and FXS. On the other hand, FXS and ASD have different genetic causes, so FXS patients' cytokine profiles may show unique signatures that differ from ASD.

Our results showed that some pro-inflammatory cytokines were elevated in FXS patients. Pro-inflammatory cytokines, like IL-1, can alter proliferation, cell survival, cell death, neurite outgrowth, and gene expression in neurons (Gadient and Patterson, 1999; Mehler and Gokhan, 1999). Persistent production of pro-inflammatory cytokines, especially in early development, leads to neuronal damage and elicits a series of neurodevelopmental deficits. In animal research, prenatal murine exposure to LPS resulted in decreased prepulse inhibition of the acoustic startle reflex (Borrell et al.,

2002). In ASD research, cytokine dysregulation during pregnancy was shown to influence immunological profiles and neurobehavioral patterns of the offspring (Ponzio et al., 2007). In FXS research, It has been shown that autoimmune disease in *Fmr1* premutation mothers is associated with seizures in their children with FXS (Chonchaiya et al., 2010).

2. Cytokines alterations specific to FXS.

Several cytokines, like TNF, IL-27, IL-8, RANTES and GM-CSF did not show significant difference in ASD patients' samples in any published literature, and could be specific to FXS.

One cytokine, TNF, showed significantly higher basal levels in plasma from FXS patients but not from ASD patients when comparing with TD controls. TNF has been linked with neurite growth and the regulation of homeostatic synaptic plasticity in the hippocampus (Cacci et al., 2008) , while TNF administration inhibits long-term potentiation (LTP) in young mice (Cunningham et al., 1996). These properties may be related to the symptoms of FXS.

TNF has an AU-rich element (ARE) with in the 3'UTR of its mRNA (Zhang et al., 2002). Khera et al demonstrated that, FXR1, a close homolog of FMRP, is associated with TNF mRNA and mediates TNF translation (Khera et al., 2010). Macrophages from FXR1 knockout mice had enhanced TNF protein production compared with wild type following activation (Garnon et al., 2005). Since FMRP can also bind to AREs, TNF expression might post-translationally regulated by FMRP. In FXS patients, the

suppression mediated by FMRP is missing, giving rise to enhanced TNF cytokine production and elevated protein levels in plasma

IL-27 is a heterodimeric cytokine belonging to the IL-12 family (Rousseau et al., 2010). It's a potent inhibitor of a newly found T helper cell subset, Th-17 (Amadi-Obi et al., 2007). Recent studies suggested that Th-17 cells may be involved in development of autoimmune and inflammatory disease, while IL-27 is neuroprotective against inflammation mediated by Th-17 and other TNF producing cells (Giuliani and Airoldi, 2011). IL-27 receptor–deficient mice chronically infected with *Toxoplasma gondii* developed severe neuroinflammation. Furthermore, IL-27 has been shown to inhibit Oncostatin M TNF-alpha and iNOS expression at the transcriptional level and rescue the neurotoxicity induced by OSM-stimulated microglia (Baker et al., 2010). In FXS , lower IL-27 production might lead to an elevated immune response in those patients.

3. Chemokines.

We observed changed levels of 3 chemokines (IL-8, RANTES and GM-CSF) in FXS. This was not observed in ASD in the published literature; however chemokines are unstable and may have been missed in previous analysis.

Chemokines are a family of small cytokines, and usually act as chemoattractants to guide cell migration. These three chemokines mentioned above are closely associated with inflammation. In the peripheral and central nervous system (CNS), secretion of chemokines recruits inflammatory cells and induces oxidant stress. Recent research demonstrated that, In CNS, chemokines are actively involved in the

communication between neurons and microglia; their chemoattractant properties function as cues for the migration of newly generated neurons/glia as well as modulators for axon pathfinding (Deverman and Patterson, 2009). High chemokine levels during certain developing periods are associated with increased impairments in behaviors. It has been reported that pregnant mothers with high levels of interleukin-8 have increased risk of schizophrenia in their offspring (Brown et al., 2004).

Although we have demonstrated altered cytokine/chemokine profiles in FXS, the precise role those cytokines played during neurodevelopment and how those cytokines contribute to the neuropathology in FXS is still not clear. However, our observations may provide critical evidence to justify a focus on immune dysregulation in FXS, by clinical investigators.

## Figures

### Figure 3.1 Genes in “inflammatory responses” gene set

A large portion of the genes actually encode cytokines. On the heatmap (red to blue scale), red means the gene expression is higher than average, while blue means the gene expression is lower than average. A large portion of the genes in “inflammatory responses” gene set were up-regulated in FXS (FMR1-8), comparing with controls (NORM1-15).

## Figure 3.1 (cont.)

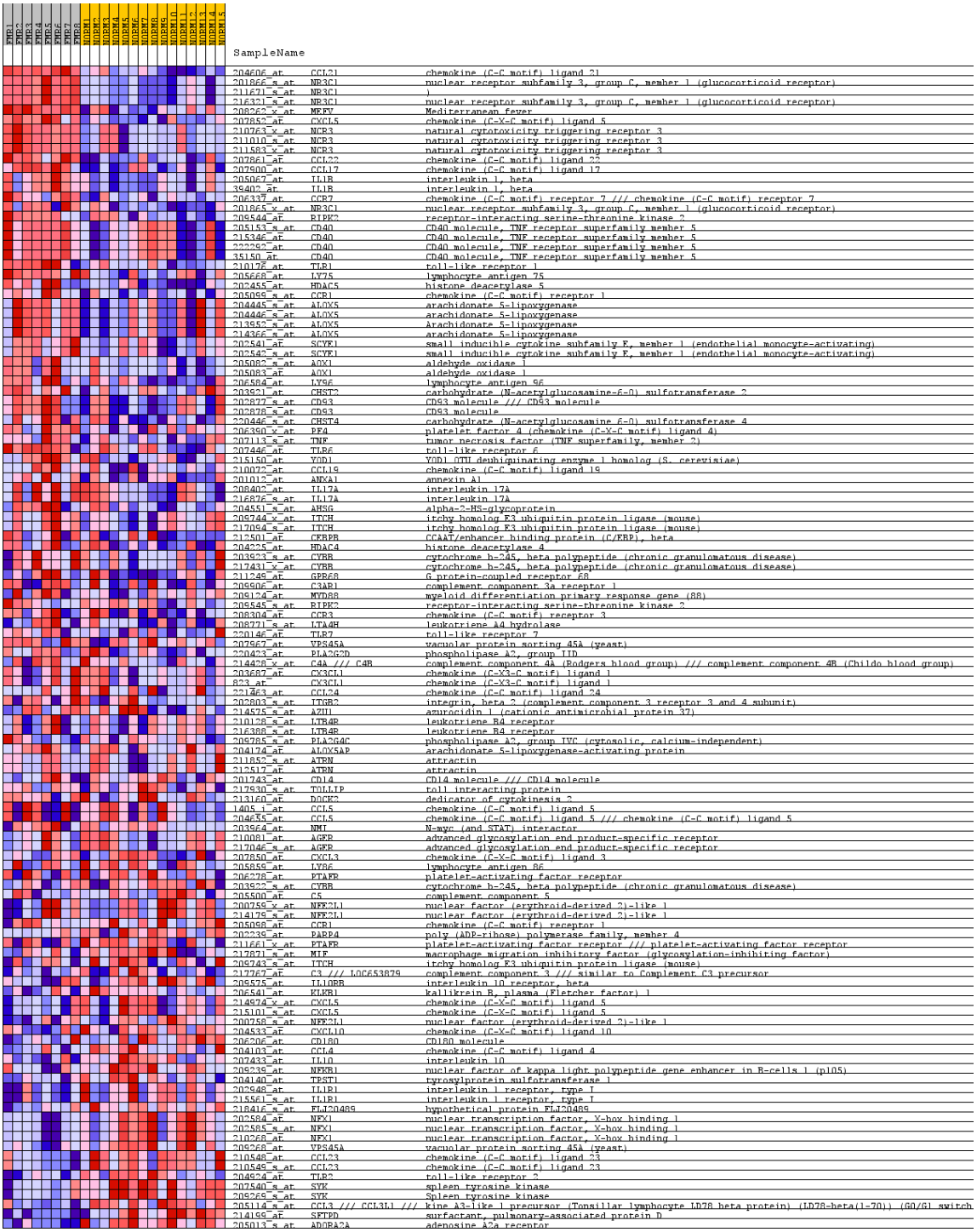


**Figure 3.2 Plasma cytokine profiles**

Sample plasma was collected within 24 hours after blood samples are taken. The relative concentration of the cytokines was measured. Out of 36 cytokines, significant differences were observed from IL-1β, IL-27 and TNF-α and IL-8.

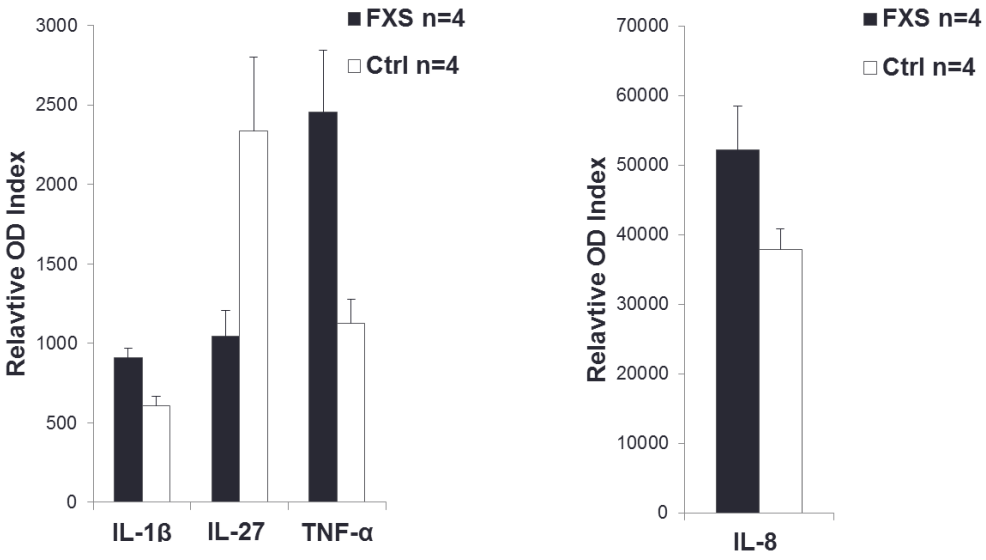


**Figure 3.3 Active Cytokine production**

PBMCs were washed and cultured in serum-free medium for 16 hours, the concentration of cytokines was assayed by measuring the cytokine concentration in culture medium. Significant differences were observed on IL-1β, IL-1ra, IL-8, TNF-α, RANTES and IL-27.

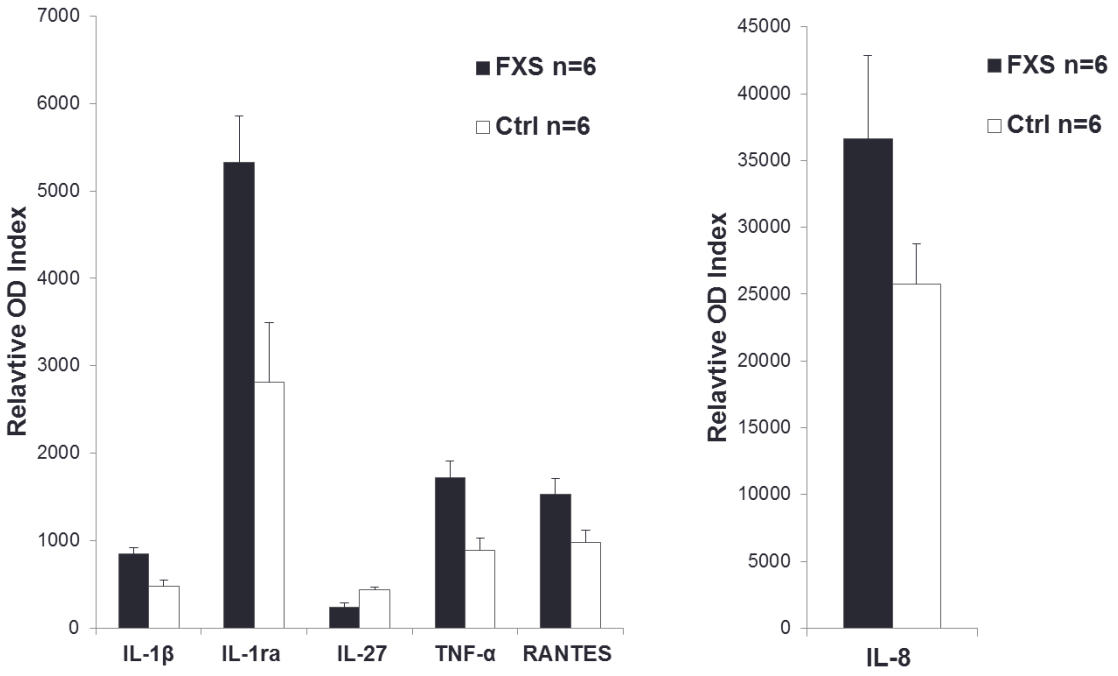
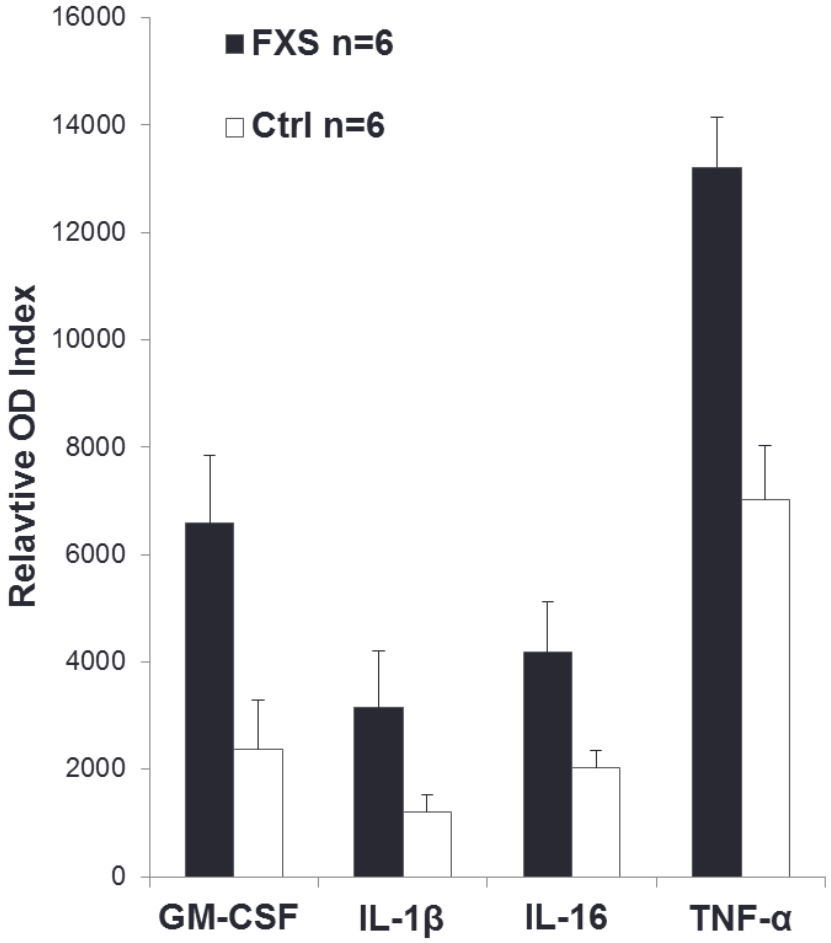


**Figure 3.4 LPS-induced cytokine release**

LPS potently triggered pro-inflammatory cytokine release. After 16 hours in LPS, PBMCs from FXS patients produced significantly higher levels of GM-CSF, IL-1β, IL-16 and TNF-α than TD controls.



# CHAPTER 4

# CONCLUSIONS

The ERK biomarker assay is the first non-invasive test for FXS patients. It is designed to be used by regular clinical laboratories with basic molecular biology equipment and single-laser flow cytometers. The ERK biomarker assay can tolerate certain degree of cell death during processing (sample viability rate should be greater than 80%), because the irresponsive cells can be excluded on flow cytometers. On a flow cytometer, the ERK signal is standardized by cell number and is independent of sample loading that could introduce variance. Using the time ERK activation reaches half maximum level, the final result is not sensitive to slight difference in the staining pERK antibody used between tests and the laser compensation settings between flow cytometers in multiple clinical sites. The ERK biomarker assay is quite accurate, the difference between repeated measurement of the same blood sample is usually less than 0.1 minutes. In the Riluzole clinical trial, subject 3 and subject 4 are monozygotic twins, their ERK scores were almost identical in our blind test.

The efficacy of the ERK assay has been proven by several clinical trials. However, there are some limitations that should be considered when applying the assay in clinical research.

First, female patients are usually less affected by FXS than male patients, because in most of the cases, females have a functional copy of the *Fmr1* gene on the other X chromosome. In addition, “mosaic” FXS patients are partly affected, the FMRP is

expressed in some, but not all tissues. ERK biomarker scores of the female and the mosaic FXS blood samples have no statistically significant difference when comparing with normal controls. Secondly, if a pharmaceutical agent can directly alter ERK phosphorylation (though MEK activation), treatment with the pharmaceutical agent can change the T1/2 number by shifting the ERK activation curve, but the change may not reflect the actual change in behavior. Besides, shipping conditions can significantly affect sample quality and eventually change ERK score (Figure 2.6). A temperature-controlled, shock-absorbing, double-sealed container should be used for overnight shipment.

The ERK biomarker assay requires only 2-3 ml blood sample for each test. This is usually not a problem for human FXS subjects. The assay can be adapted to animal model in pharmacological research with minor adjustment. In *fmr1* KO mice, 500 µl blood sample is enough to measure pERK levels of 5 time points, which are sufficient to generate an ERK activation curve.

Overall, our pilot work on the ERK biomarker assay has the potential to be used in both clinical and pre-clinical research of FXS.

Our research on cytokine is the first of its kind to reveal the dysregulated immune profiles in FXS. Dysregulated cytokine profiles could be active determinants of the FXS symptoms. It has been reported, the prevalence of autoimmune diseases are higher in females with the *Fmr1* premutation. Maternal autoimmunity could contribute

to autism or autistic-like behavior, as well as other psychiatric symptoms in their children with FXS. On the other hand, altered cytokine profiles could be a result of the abnormal protein translation control due to the loss of FMRP. For example, FMRP could regulate TNF expression by associating AU-rich elements in TNF mRNA; without FMRP, the precise translation control on TNF expression in the immune cells (macrophages or microglia) could be absent. Elevated TNF production may be implicated in the pathology of FXS.

Although our research is limited by the total number of FXS patients we could recruit, the result still elicits concerns for clinical investigators when treating FXS patients, and should be considered when designing therapeutic agents for FXS subjects.

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