Title
Mechanisms Underlying Neurodegeneration in Multiple Sclerosis: A Region-Specific, Cell-Specific, and Sex-Specific Approach

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Mechanisms Underlying Neurodegeneration in Multiple Sclerosis: A Region-Specific, Cell-Specific, and Sex-Specific Approach

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Lisa Golden

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ABSTRACT OF THE DISSERTATION

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A Region-Specific, Cell-Specific, and Sex-Specific Approach

by

Lisa Golden

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2020

Professor Rhonda. R. Voskuhl, Chair

Like most autoimmune diseases, multiple sclerosis (MS), is more prevalent in women than men, thought to be due to more robust immune responses in women. The effects of sex hormones on the immune response have been extensively studied by our lab and others, but effects of sex chromosomes are widely unknown. Sex chromosomes can elicit gene expression differences between males and females via expression of Y genes, X gene dosage effects, or parent-of-origin differences in DNA methylation of X genes, also called parental imprinting. We previously showed that sex chromosomes modulate immune responses in the animal model of MS experimental autoimmune encephalomyelitis (EAE), however, the mechanisms remained unknown. Chapter 2 of this dissertation identified parent-of-origin differences in DNA methylation of X chromosome genes as a mechanism by which sex chromosomes alter gene expression in immune cells. Through a series of RNA sequencing experiments using the “four core genotypes” model, we discovered a cluster of X genes with higher expression in XY than XX genotypes in CD4+ T lymphocytes. Additionally, DNA methylation studies in X monosomic mice revealed parent-of-origin differences in DNA methylation in which the paternal X is hypermethylated compared to the

maternal X and autosomes, which is consistent with higher expression of X genes in XY compared to XX. This work demonstrated how parental imprinting of X chromosome genes can lead to sex differences in gene expression during immune responses.

While women are more susceptible to MS, disability progression tends to be worse in men, thought to be due to a more neurodegenerative response to immune attack. This identifies males as a superior target for understanding mechanisms of neurodegeneration. In Chapter 3 of this dissertation, I take a cell-specific and region-specific gene expression approach to investigate the neurodegenerative response in the hippocampus during injury. Through transcriptomics studies in hippocampal astrocytes, I found upregulated expression of genes involved in MHC class I (MHC I) signaling. Since MHC I has an important role in synaptic plasticity, I hypothesize that astrocyte MHC I signals to microglia and neurons to induce aberrant synaptic pruning in adult mice with disease (EAE). I will test this hypothesis with time- and cell-specific conditional knock out of *B2m* to reduce MHC I cell surface expression in astrocytes during EAE. Additional studies utilizing gonadectomy will determine the effects of sex hormones on MHC I gene expression.

Together, these projects seek to fundamentally understand complex mechanisms in MS susceptibility and progression, and how sex affects these aspects of disease. Further understanding of these mechanisms may lead to identifying natural disease modifiers and new translatable targets for neuroinflammatory therapeutics.

The dissertation of Lisa Golden is approved.

Arthur P. Arnold

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University of California, Los Angeles

2020

This dissertation, as well as every day, is dedicated to my mother Donna Golden.

My inspiration, my motivation, my guardian angel.

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Author. Bench to Bedside: Integrating Sex and Gender to Improve Human Health. Immunology. **2019**. https://orwh.od.nih.gov/career-development-education/e-learning/bench-bedside

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Chapter 1

Introduction:

The Importance of Region-Specific, Cell-Specific, and Sex-Specific Experimental Approaches in Neurodegenerative Disease

1.1 Multiple Sclerosis: An Autoimmune Disease of the Central Nervous System.

Multiple sclerosis (MS) is a complex disease involving both the immune system and the central nervous system (CNS). In MS, T lymphocytes are activated and migrate through the blood brain barrier (BBB). These T-cells release cytokines that activate microglia and astrocytes, as well as recruit macrophages and other lymphocytes leading to demyelination and irreversible neurodegeneration4. There are several different types of MS. Relapsing-remitting MS (RRMS) is characterized by episodes of autoimmune infiltration inducing a new symptom or significantly worsening a pre-existing symptom, each known as a relapse. Relapses are followed by a period of autoimmune quiescence and clinical stability, or, remission. Over decades, RRMS often transitions into secondary progressive MS (SPMS). MS patients that no longer exhibit relapses and remissions but instead slowly accumulate worse neurodegeneration, have SPMS. Approximately 10% of MS patients do not have an initial relapsing phase that precedes the neurodegenerative phase, and this is termed primary progressive MS (PPMS)

1.2 The Importance of Region-Specific and Cell-Specific Approaches in Neurodegenerative Disease.

MS is a multifocal disease, meaning patients have multiple inflammatory lesions within the CNS. The CNS can be broken down into many functionally distinct regions. At the simplest level, the CNS includes the brain and the spinal cord. Within the brain, there are myriad regions such as the cortex, hippocampus, and cerebellum to name only a few, which in turn have subregions. For example, the cortex can be further divided into frontal, motor, visual, and sensory cortex, while the hippocampus can be further divided into dorsal and posterior hippocampus, or into subregions such as the CA1, CA2, CA3 and dentate gyrus. Each of these regions and subregions of CNS tissues serve in distinct physiological functions. Therefore, neurological damage in a given region results in specific disabilities correlating with the function of that region[1]. For this reason, not all MS patients have the same disabilities.

Within these functionally distinct CNS regions are several cell types, all of which have unique roles in CNS homeostasis and response to inflammatory stimuli. This can confound the assessment of changes in important genes and pathways in any one cell type with whole tissue analyses. Additionally, changes in whole tissue gene expression can be representative of changes in cell composition due to proliferation, migration, and cell loss during disease. Therefore, it is important to study each individual cell type separately. We compared gene expression from spinal cord whole tissue (i.e. all cell types) to that of only spinal cord astrocytes during neurodegenerative disease using the pre-clinical model for MS, experimental autoimmune encephalomyelitis (EAE). We found clear differences in gene expression between whole tissue and astrocytes that demonstrated the importance of cell-specific gene expression analyses[2]. Further, neurons, astrocytes, microglia and oligodendrocytes have all been shown to have physiological diversity in different brain regions[3-7] to accommodate the specific functions and environment of that region. We recently showed that astrocytes have regional gene expression diversity during disease, again using EAE[2]. This suggests that the mechanisms of disease may differ between CNS regions. Thus, understanding molecular mechanisms in a region-specific and cell-specific manner may uncover previously overlooked molecular mechanisms of disease and lead to disability-specific targets for therapeutic intervention.

1.3 The Importance of Studying Both Sexes; an Overview of Sex Differences in Neurodegenerative Disease.

To date, scientific research has often focused on one sex, with assumptions that study of the other sex would yield similar results. However, many diseases affect males and females differently, and understanding mechanisms underlying sex differences in disease can lead to the discovery of natural disease modifiers. It is important to study both sexes for many reasons. The sex of a patient often changes the risk for disease susceptibility or progression by fold differences, not mere percentages. In addition, optimal dosing of treatments, considering efficacy and side

effects, can differ between the two sexes. Optimizing the dose for one sex and assuming findings are no different in the other sex is underserving a large portion of patients. The risk:benefit ratio for a given dose must be determined for each of the two sexes. Finally, the study of both sexes may lead to the discovery of unexpected natural disease modifiers.

Researchers studying the MS model EAE found that female mice, rats, rabbits and guinea pigs exhibited protection from disease when they were pregnant[8, 9]. Such an observation would never have been made if only the male population were studied. Since it had been anecdotally observed that there were protective effects for women with MS when they were pregnant, this observation in animals suggested that the improvement of disease in pregnant MS women was due to an important biological disease modifier of pregnancy, not merely an effect of differences in reporting or other circumstances of women during pregnancy.

When researchers included both females and males in adoptive transfer EAE experiments, they found that females, as compared to males, were more susceptible to disease in the relapsing remitting model[10]. Since epidemiologic studies showed that men were less susceptible to MS than women, these preclinical studies again supported a biologic mechanism for the sex difference in MS, not mere differences in MS reporting or perception of symptoms.

1.4 Sex Differences in MS.

MS exhibits sex differences in both the immune response and neurodegeneration, affecting disease susceptibility and progression, respectively. However, sex differences in the immune system appear to be different than sex differences in the CNS; women are more susceptible to MS[11], but men are more likely to have disease progression[12-14]. This poses the question, if the incidence of disease is higher, and peripheral immune responses are more robust in women, then why isn't disability progression faster in women? If anything, there is faster progression in men. We have hypothesized that sex related factors could play different roles in the immune system versus the CNS, and thus different roles in susceptibility versus disability progression[15].

Sex Differences in MS Susceptibility (Female > Male)

Disease onset

There is a distinct female preponderance to a variety of autoimmune diseases including MS, systemic lupus erythematosus, and rheumatoid arthritis[11]. Consistent with increased susceptibility in females are reports that immune responses are more robust in healthy females compared to males, including autoantigen specific responses in MS[16-19]. The female to male ratio in MS varies somewhat by geographic region from 2:1 to 3:1, and has increased in the past decades with a few notable exceptions[20]. This increase in the female to male ratio is driven by increased incidence in women, not by decreased incidence in men. The reasons for the increased incidence in women remain speculative. However, the short time span over which it is occurring excludes a purely genetic cause[21]. Instead, it has been proposed to be due to gene-environment interactions or epigenetic factors[15, 22].

Relapsing-Remitting MS

While the female preponderance for RRMS has been established for many years[23], a recent study demonstrated a 17.7% higher relapse rate in females compared with males who have RRMS throughout the entire duration of disease and at any age[24]. Also, RRMS patients have a greater female to male ratio when they have at least 4 relapses per year in the first five years of disease, (3.3:1) than patients who experience no relapses in the first five years (2.3:1)[24]. Females also have a higher cumulative hazard risk of relapses than males 40 years after disease onset[24]. Conversely, there is no distinct sex difference in primary progressive MS patients (1.2:1), the type of MS without relapses[24].

Pre-clinical model for relapsing-remitting MS

EAE in the SJL strain of mouse exhibits a relapsing-remitting pattern[25]. Therefore, this model has been used extensively to study immunological mechanisms of relapses in MS by focusing on myelin specific immune responses in lymph node and spleen, inflammation and

demyelination in spinal cord, and clinical EAE score that principally reflects walking disability. Females as compared to males are more susceptible to EAE in the SJL strain[10].

Sex Differences in MS Disability Progression (Male > Female)

Disease Progression in RRMS Patients

For years it was reported that early predictors of future permanent disability in MS included male sex, age of onset, and degree of recovery from the first episode[26]. A large natural history study of all MS subtypes showed that the median time between disease onset and reaching a given disability level was shorter in men[27]. Other studies also showed that being male was associated with shorter time to disability progression in RRMS[28-30]. These publications addressing changes in long term disability progression were in contrast to findings of no sex difference in disability progression during short term trials testing anti-inflammatory treatments for effects on relapses. A natural history study of untreated MS patients found that the male sex was associated with a shorter time to, and a younger age for, conversion to SPMS[13]. Another study showed that males have a more severe disease phenotype with faster accumulation of disability[12], with yet another showing that male sex and older age at onset were predictive of more rapid progression from disease onset of RRMS[14]. Finally, A very large registry-based study of over 14,000 patients revealed that male relapse-onset progressive patients accumulated disability faster than female relapse-onset progressive patients[31].

While the above summarizes sex differences in several MS disabilities as measured by composites, cognition is a specific disability that appears to be worse in men. An early study showed that male MS patients performed worse on several cognitive subtests as compared to female MS patients who were matched for age, education, and other neurologic and emotional measures[32]. Later, a large cohort of MS subjects (n = 533) were categorized as cognitively normal or impaired based on their performance on a battery of neuropsychological tests. These subjects

also underwent genotyping for the APOE-epsilon4 allele polymorphisms. Cognitive decline was more prominent in men and was associated with the presence of the epsilon4 allele of the APOE gene[28]. To investigate the relationship between subcortical GM atrophy and cognition in early relapsing onset MS, structural MRI and neuropsychological testing were performed in 80 MS women and 40 MS men, as well as in 30 healthy women and 20 healthy men. Several deep GM structures showed volume loss in MS compared to healthy controls, with larger effects in MS men (-11%) than in MS women (-6.3%). Also, more cognitive domains were affected in men than in women[33]. Among the subcortical GM structures assessed, it was thalamic atrophy which showed the closest association with cognitive impairment.

Primary Progressive MS

It has long been known that the characteristic profile of primary progressive MS (PPMS) is being male, being older and having spinal cord lesions[34]. A recent study aimed to identify predictors to develop PPMS in a population of subjects with radiologically isolated syndrome (RIS). RIS is the term used to describe the presence of white matter lesions not associated with other clinical symptoms[35]. It was found that male sex, older age, and lesions predominantly in spinal cord were the three predictors of evolution from RIS to PPMS[36]. However, during the primary progressive disease course, males do not have significantly faster disease accumulation than women, contrary to their faster disease accumulation in RRMS disease course[31].

Pre-clinical model for neurodegeneration

As stated above, EAE has historically been used to study immune responses, walking disability, and spinal cord pathology. However, over the past decade, EAE has been shown to also include neurodegenerative pathology beyond spinal cord, particularly in the chronic EAE model in the C57BL/6 strain. The cerebellum has white matter inflammation, demyelination and axonal loss as well as Purkinjie cell loss[37, 38]. The hippocampus is also affected during EAE, displaying CA1 atrophy and loss of synaptic integrity, the latter of which correlated with altered

functional synaptic transmission using electrophysiology of hippocampal slices [39-41]. Cerebral cortical structures in EAE show synaptic loss, transected axons, and loss of callosal projecting neurons and pyramidal neurons[37, 42, 43], while the corpus callosum has areas of demyelination, with decreased axon conduction by electrophysiology[42, 44]. Additionally, the striatum is affected by altered excitatory transmission, again associated with synaptic degeneration and dendritic spine loss[45]. Studies of optic neuritis also use EAE to show loss of retinal ganglion cell bodies and optic nerve axons. In addition to pathological abnormalities in EAE brain tissue, whole brain atrophy[43, 46, 47] and gray matter atrophy in cerebellum and cortex have been seen[46, 48]. These neuroimaging abnormalities in EAE occur in a setting of T2 lesion hyperintensities in brainstem and cerebellum[38, 48, 49].

1.5 Unraveling Mechanisms Underlying Sex Differences in Disease

Bedside to Bench to Bedside approach

Sex differences in disease are widespread. Many of these differences were first observed at the clinical bedside, and then brought to the laboratory bench to be mechanistically dissected. These findings can then lead to novel treatments for clinical trials. This is the “bedside to bench to bedside” approach[15]. Using this approach, sex differences in disease can serve as clinical observations that provide a foundation known to be physiologically relevant in humans, as opposed to starting with a molecule of interest in preclinical models without known physiological relevance. The “bedside to bench to bedside” approach in drug development is a conceptual shift, since usually the molecule comes first, with questions of physiologic relevance coming second.

Sex Hormones and Sex Chromosomes in Sex Differences

Sex differences in any disease may be due to sex hormones, sex chromosomes or both since a role for one is not mutually exclusive of a role for the other[50]. Regarding sex hormones, physiologic levels of cycling estradiol and progesterone could play a role in females. Likewise, physiologic levels of testosterone in males could play a role. Regarding sex chromosomes, the

XX sex chromosome complement could play a role in females or the XY complement in males. These factors may act individually, or in conjunction with each other. Sophisticated approaches can now discern between these possibilities.

1.6 The Role of Sex Hormones in Sex Differences in MS

Testosterone

Clinical observations

As previously stated, men have demonstrated worse disease progression compared to females[12-14, 26-29, 31]. This is likely due to factors other than testosterone since clinical data from converging lines of evidence from independent sources suggests that testosterone is protective in men with MS. A retrospective cohort study was conducted using analysis of English National Hospital records from 1999-2011. It found that men with testicular hypofunction, a proxy for low testosterone levels, were at increased risk to develop MS, suggesting an immunosuppressive role of testosterone[51, 52]. In line with these results, patient reports show that men with MS have relatively low testosterone levels compared to healthy men[53-55]. Among men with MS, those with lower testosterone levels had worse EDSS scores, and when followed longitudinally, those with lower testosterone levels showed more decline on longitudinal cognitive testing (as assessed by the SDMT)[53]. These results suggest possible neuroprotective effects of higher levels of endogenous testosterone. While it is possible that low testosterone levels are an effect of disease, the above study reporting testicular hypofunction in patients prior to their development of MS suggested that low testosterone in MS men was not merely a consequence of disease[52].

Pre-Clinical Studies

Preclinical data has revealed a protective effect of testosterone in two different MS models, EAE[56-59] and cuprizone[60, 61]. IN EAE, testosterone treatment decreased EAE scores of walking disability, reduced inflammation and demyelination, and spared axons[56, 57, 59]. As reviewed above, in addition to walking disability, EAE in C57BL/6 mice involved cognitive

dysfunction. Testosterone treatment has been shown to be protective when assessing cognitive outcomes during EAE. Specifically, testosterone treatment induced an increase in synapses by neuropathology and improved synaptic transmission by electrophysiology[40]. Synaptic stripping and loss is observed in MS cortex[43, 62], and testosterone treatment in cognitive neurodegenerative models improves structure and function of dendrites and synapses in cerebral cortex[63]. Together these reports suggest a mechanistic hypothesis whereby testosterone treatment in MS may increase synapses and their function in cortical gray matter. Effects of testosterone treatment may be mediated through direct binding of testosterone to the androgen receptor. Alternatively, testosterone can be converted to estrogen in the brain by aromatase to mediate neuroprotection[64-66]. This hypothesis is consistent with the ability of estrogen treatment to increase dendritic spines, synapses and cognitive function in preclinical models of cognitive decline[67-72]. Beneficial effects on synapses are not mutually exclusive of additional potential effects of testosterone treatment in MS on remyelination of white matter[60] or immunomodulation[56, 59, 73]. Indeed, many complementary pathways may ultimately be involved in testosterone-mediated neuroprotection.

Testosterone mediated neuroprotection has also been shown in other preclinical cognitive neurodegenerative models including mild cognitive impairment, Alzheimer's Disease, and Apolipoprotein E induced cognitive impairment[74-79]

Clinical Trials

A single arm crossover pilot clinical trial of testosterone treatment in men with MS revealed beneficial effects of treatment on brain atrophy, muscle mass, and immune markers. Ten men with RRMS participated in an open-label phase 2 trial. Subjects were observed prior to treatment for 6 months, followed by testosterone treatment for another 12 months. This treatment increased serum testosterone levels from the low-normal to high-normal range[80]. Brain MRI's were obtained every month. Compared to the pretreatment phase, there was

slowing in the rate of whole brain atrophy by 67% during testosterone treatment[80]. Whole brain atrophy in MS is driven by gray matter atrophy, and gray matter atrophy has been used as a biomarker for permanent disability[81-87]. Thus, further analysis of the same MRIs by other investigators focused on gray matter. It was found that testosterone treatment significantly reduced gray matter atrophy as compared to pretreatment [88]. In vivo cell-mediated immune responses were decreased, as measured by delayed type hypersensitivity (DTH)[80], and peripheral blood mononuclear cell (PBMC) analyses revealed modest immunomodulatory properties with interesting increases in neurotrophic growth factor production during testosterone treatment[89]. Cognition was also improved with testosterone treatment as seen by increased PASAT scores as well as increased spatial memory[80]. However, this could have been confounded by a practice effect of repeated testing.

While any one of these observations in isolation could be considered merely suggestive, consideration of all supports the hypothesis that testosterone supplementation in men with MS, boosting them from the low- normal range to the high-normal range, may be protective[80, 88, 89]. Thus, a larger phase 2, multicenter, placebo-controlled trial of testosterone is now warranted in men with MS.

Estradiol and Progesterone

Clinical Observations

The onset of puberty correlates with an increased onset of disease in women, but not men[90]. An MRI study of relapsing-remitting MS patients revealed an increase in number and volume of enhancing lesions during the week leading up to the first day of the menstrual period[91]. However, no increase in clinical relapses has been noted during menopause or in low estrogen times in the menstrual cycle. A study on the effects of oral contraceptives on disease incidence in women showed no significant relationship[92]. These studies suggest that physiological cycling

levels of estradiol and progesterone have unclear effects on disease onset and relapses in RRMS patients.

Circulating levels of ovarian hormones during a woman's menstrual cycle have correlated with changes in pre-existing symptoms prior to the menstrual period, a time when estradiol and progesterone levels are low[93, 94]. Patients using oral contraceptives, containing both estrogen and progesterone, no longer had worsening of symptoms during the pre-menstrual period[94]. Additionally, near 50% of post-menopausal women reported a worsening of symptoms[93]. Post-menopausal women who were taking hormone replacement therapies reported an improvement in their symptoms[93]. Notably, along with biological changes, many women undergo social and environmental changes once they are post-menopausal, which complicates direct correlations with hormones in observational studies[95]. One report showed that patients who started using oral contraceptives after disease onset experienced a milder disease course as compared to those who used oral contraceptives before disease onset or never used them[96]. In another study, RRMS women who used oral contraceptives were less likely to develop secondary progressive MS[97]. However, a different study showed that those who used oral contraceptives had a higher risk of reaching EDSS score of 6[98]. Overall, the effects of physiological levels of circulating ovarian hormones on MS symptoms and disability progression remains unclear.

Pre-Clinical Studies

Pre-clinical studies ovariectamize (OVX) rodent MS models to study the effects of adult sex hormones on disease. The results from several studies leave the effects of ovarian hormones on EAE disease course controversial. Some studies show that OVX leads to earlier onset and worse clinical disease compared to sham controls[99, 100], while another group reported no difference in EAE disease course between OVX and sham controls[101]. One study in rats also showed worse disease in OVX females[102].

Ovarian hormone treatments in EAE showed that treatment with estradiol ameliorated EAE clinical disease severity in both intact and OVX animals[99, 103-105]. A dose response was shown for estradiol whereby higher doses were more protective[105]. Pregnancy levels of estrogens were clearly protective, while lower levels were modest. Progesterone alone has been shown to have only mild effects on EAE clinical disease in C57BL/6 mice. Progesterone treatment decreased EAE clinical disease severity and decreased demyelinating lesions in spinal cord[106-108]. However, when progesterone treatment was administered to SJL female mice, no significant effects on EAE were observed[109]. Ovarian hormone treatment in rats resulted in slight alleviation of disease with estradiol treatment, and worsening of disease with progesterone treatment alone[102]. However, in mice and rats, combined treatment with estradiol and progesterone had greater amelioration of disease than estradiol alone, indicating an additive effect of progesterone to estradiol[102, 107].

Clinical trials

The effects of oral contraceptives were studied prospectively in a clinical trial. The study used the standard of care, IFN-β, in combination with either low or high doses of ethinylestradiol in combination with the progestin desogestrel in a 96-week trial. Significant decreases in the cumulative number of combined unique active lesions and Gd-enhancing lesions were seen in patients receiving the high dose, however this was not the primary outcome measure. No other MRI outcomes displayed significant results in either dosage group. No difference on relapse rate, the primary outcome, was observed[110]. Further trials of high dose estradiol containing oral contraceptives in MS are lacking. Increased risk of breast cancer must be considered in such trials since estradiol binds strongly to estrogen receptor alpha (ERα), the mediator of adverse events with oral contraceptives and hormone replacement therapy[111].

The Protective effects of Pregnancy in Females with Relapsing-Remitting MS

Clinical Observations

Disease is ameliorated during late pregnancy in both MS and the MS model experimental autoimmune encephalomyelitis (EAE)[8, 112]. A landmark study showed that there was over 70% reduction in MS relapses during the third trimester of pregnancy[112]. Several groups then began investigating which biological factor of late pregnancy might mediate this protection from relapses at the bench. Many hormones are increased during late pregnancy, including estriol, estradiol and progesterone, that could contribute to the protective effects of pregnancy on MS. Estriol is unique to pregnancy in that estriol levels are absent in women during non-pregnancy, but are made by the fetal placental unit, and reach high levels during the third trimester, the time when a 70% decrease in relapse rate was reported[112]. Estriol was therefore studied for its protective role during EAE in preclinical studies. Estriol is also known to be the safest of the estrogens for decades[113, 114]. ERα has been implicated in promoting breast cancer, while ERβ has been shown to be less toxic, potentially inhibiting breast cancer[115]. Estriol binds ERβ with higher affinity than ERα[116].

Pre-clinical studies

Investigation of the effects of estriol treatment in the EAE model showed amelioration of disease severity compared to those treated with placebo when treatment was given either before or after disease induction and in several EAE models[105, 109, 117]. Pathology revealed less inflammation, less demyelination, and sparing of axons in spinal cord in estriol treated animals compared to placebo[109]. Estriol treatment also resulted in sparing of hippocampal CA1 volume along with preservation of synapses and reduced microglial activation[72]. This could be due to estriol acting on either estrogen receptor (ER) α or ERβ, since both have been implicated in estrogens' protective effects in EAE[118-120]. ERα mediated protection effects all stages of disease, while ERβ mediated protection effects the chronic stage of the disease[121]. This is consistent with reports of ERβ being expressed widely in the brain[118], potentially acting though microglia,

oligodendrocytes, or dendritic cells[122-125]. Protection against disease was also seen in males treated with estriol, not only females[126]. As described above, therapeutic effects of estradiol and progesterone were also investigated since they could also contribute to disease improvement in late pregnancy.

Pregnancy is protective for Th-1, not Th-2, mediated immune diseases, due to pregnancy skewing the immune response to Th-2 so as not to reject the fetus[127]. Therefore, estriol may be beneficial for other Th-1 mediated immune diseases such as psoriasis and rheumatoid arthritis.

Clinical Trials

Since estriol was protective in EAE, clinical trials in women with MS began. A small pilot Phase 2 trial showed a 70-80% reduction in inflammatory lesions in white matter on brain MRI[128] and peripheral blood mononuclear cells showed favorable immunomodulation[129]. A larger phase 2, randomized, placebo-controlled trial of estriol versus placebo was conducted in relapsing remitting MS (RRMS) women taking standard of care MS treatment (glatiramer acetate, GA). The study hit its primary outcome measure of relapse reduction. Specifically, when these patients were given estriol in addition to the standard of care, there was a one-half to one-third further reduction in annualized relapse rates over and above that provided by standard of care treatment[130]. Promising effects on exploratory outcomes showed improved cognition and decreased fatigue, with slowing of cortical gray matter atrophy on brain MRI[130]. A phase 3 trial is needed to confirm the benefit of estriol treatment in women with RRMS.

The other potential hormone of pregnancy could be progesterone. There is a remarkable increase in relapse rate post-partum, when progesterone and estrogen levels decrease dramatically[112]. A French group followed up on this observation with a clinical trial treating post-partum women with progesterone, aiming to decrease relapse rate as the primary outcome. The results from this study have not been published to date, but the study was terminated when it

became clear that it would not hit its primary outcome (ClinicalTrials.gov Identifier: NCT00127075).

While a protective role of estriol in MS is not mutually exclusive of other pregnancy factors also playing a role, twenty years after the observation of the decreased relapses in women with MS during the third trimester, no pregnancy factor other than estriol has shown efficacy in a Phase 2 trial. Thus, other autoimmune diseases known to improve during pregnancy, such as psoriasis and rheumatoid arthritis[11], should also be investigated for a protective effect of estriol at a dose recapitulating a pregnancy level.

1.7 The Role of Sex Chromosomes in Establishing Sexual Dimorphisms in MS

As described above, there have been extensive studies investigating the role of sex hormones in MS, but this is not exclusive of an additional role of sex chromosomes. Sex chromosome differences are inherently tied to sex hormone differences since the XY genotype is associated with testes and the XX genotype with ovaries. Elegant studies have established how to determine the effect of sex chromosome complement separate from effects of gonadal type and sex hormones[131], using the "four core genotypes" (FCG) mice[132]. Specifically, the *Sry* gene on the Y chromosome that encodes for testicular development has been deleted; these mice are signified as XY^-. Since there is no *Sry* gene, these mice are, by default, ovary bearing (gonadal females). The FCG model permits comparisons between XX and XY^- mice that differ in sex chromosome complement while sharing a common gonadal type (both females throughout development). When Sry is inserted at an autosomal location, it results in XX*Sry* and XY^-*Sry*, again differing in sex chromosome complement while sharing a common gonadal type (both male throughout development).

Sex Chromosome Effects in the Immune System of the MS model EAE

To ascertain whether sex chromosomes could have direct effects on the immune system in the MS model EAE, four core genotype (FCG) mice were back-crossed onto the SJL background, a genetic background known to exhibit a greater female susceptibility[133]. Adoptive transfer of autoantigen-stimulated XX immune cells, compared to XY^- immune cells, into wild type females led to worse clinical disease and neuropathology, along with decreased Th2 anti-inflammatory cytokines such as IL-10 and IL-13[131]. These studies demonstrated a role for sex chromosomes in the induction of the immune response in adoptive EAE, with the XX complement, as compared to XY^-, more proinflammatory. A proinflammatory effect of XX compared to XY^- was also shown in experimental[131] and spontaneous[134] lupus models.

Sex chromosome effects in the CNS of the MS model EAE

An effect of sex chromosomes in the CNS response to injury during chronic EAE in C57BL/6 mice has been shown[37]. A challenge in MS models entails discerning whether effects of a treatment on neurodegeneration are merely a consequence of effects on the immune system. Therefore, bone marrow chimeras are widely used to study compartmental effects *in vivo* in EAE. This entails irradiating mice to abolish their immune system followed by reconstitution of their immune system using bone marrow from a donor mouse. Sex differences in neurodegeneration in EAE can be discerned if disease is induced in bone marrow chimeric mice that have an immune system of the same genetic sex, while having a CNS with a different genetic sex (XX or XY^-)[37]. Specifically, XX versus XY^- bone marrow chimeras were reconstituted with a common immune system of one sex chromosome complement. Using this model, EAE mice with XY^- sex chromosome complement in the CNS, compared with XX, demonstrated greater EAE clinical disease severity with more neuropathology in spinal cord (axonal and myelin loss), cerebellum (Purkinje cell and myelin loss) and cerebral cortex (synaptic loss)[37]. This result is consistent with males as compared to females having faster disability accumulation. As an example of the

importance of studying sex chromosomes, a candidate X-linked gene, Toll-like receptor 7 (Tlr7), was examined due to its role in mediating neuronal degeneration in cortical neurons[135]. Tlr7 protein expression in cortical neurons was higher in mice with XY^- compared to mice with XX CNS[37]. This was the first demonstration of a direct effect of sex chromosome complement on neurodegeneration in a neurological disease[37].

The study of sex differences in neurodegeneration in the model of MS could reveal new targets for a treatment to halt disability progression in MS. This is important since the current MS treatments target relapses, with only a modest effect on disability progression and with no approved treatments for progressive MS. Treatments to halt disability progression in MS may also warrant consideration in other neurodegenerative diseases characterized by a sex difference, including Parkinson's Disease and ALS. Parkinson's Disease is clearly male predominant as shown in a meta-analysis, where being male increased risk by 1.5 fold[136]. ALS also demonstrates a male predominance over 1.5 fold and an earlier onset of disease in men[137].

1.8 Conclusion

Anatomical region, cell type, and sex are three key factors that should be considered while studying molecular mechanisms of disease susceptibility and progression. While individual cell types are commonly investigated in the immune response, the importance of cell-specific and region-specific studies in the CNS are just coming to light. Since neurological disabilities correlate with the location of CNS injury, not all patients with neurodegenerative disease have the same disabilities. Therefore, a treatment targeting molecular mechanisms of one disability in MS may not work for other disabilities, and therefore will not be effective in every MS patient. Taking region-specific and cell-specific approaches may uncover different targets for therapeutics for individual neurological disabilities.

The importance of studying both sexes has only recenlty been appreciated in both basic and clinical research. Advancements in multiple sclerosis research underscore the need for investigating disease in both males and females. Increased susceptibility of females over males, but increased rate of disability progression of males over females, indicate gender effects on disease mechanisms. Therefore, treatments discovered for one sex may behave differently in the other sex. Additionally, by addressing both sexes, potential new treatments involving sex hormones estriol and testosterone are being pursued. Finally, addressing sex differences can reveal influences of sex chromosome genes on disease. None of these discoveries would have been possible without inclusion of both sexes in research. Examples such as these have prompted the new NIH policy requiring justification for exclusion of one sex in basic research grants[138], with implementation starting in January of 2016[139]. This new NIH policy is a win-win situation since experts not usually studying sex differences can ascertain whether their observations in one sex are also present in the other sex. If they are found in the other sex, then they will have extended their work to the other half of the population. If they are not found in the other sex, then investigators will have uncovered an interesting sex difference that could potentially lead to novel therapeutics.

Chapter 2 of this dissertation builds on the current knowledge of sex chromosome effects in the immune response to further understand mechanisms behind female susceptibility to autoimmune disease. We took a cell-specific and sex-specific approach to study gene expression differences between XX and XY genotypes in T lymphocytes isolated from draining lymph nodes. We discovered upregulation of X genes in XY genotypes, indicating a role for parent-of-origin differences in DNA methylation of X chromosome genes as one mechanism of sex differences in immune responses.

Chapter 3 focuses on understanding mechanisms of neurodegeneration in the hippocampus during EAE. I took a cell-specific and region-specific approach to study gene

expression differences in hippocampal astrocytes during chronic EAE and discovered upregulated expression of genes and pathways involved in MHC class I signaling in EAE compared to normal astrocytes. MHC class I expression in the CNS plays a critical role in synaptic plasticity, suggesting a mechanism by which astrocytes promote synaptic loss in response to injury. Interestingly, MHC I signaling genes are likely regulated by estrogen signaling, which will be investigated in future experiments.

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Chapter 2

Parent-of-Origin Differences in DNA Methylation of X Chromosome Genes in T Lymphocytes

2.1 Abstract

Many autoimmune diseases are more frequent in females than males in humans and their mouse models, and sex differences in immune responses have been shown. Despite extensive studies of sex hormones, mechanisms underlying these sex differences remain unclear. Here, we focused on sex chromosomes using the “four core genotypes” model in C57BL/6 mice and discovered that the transcriptomes of both autoantigen and anti-CD3/CD28 stimulated $CD4^+$ T lymphocytes showed higher expression of a cluster of five X genes when derived from XY as compared to XX mice. We next determined if higher expression of an X gene in XY compared to XX could be due to parent-of-origin differences in DNA methylation of the X chromosome. We found a global increase in DNA methylation on the X chromosome of paternal as compared to maternal origin. Since DNA methylation usually suppresses gene expression, this result was consistent with higher expression of X genes in XY cells because XY cells always express from the maternal X chromosome. In addition, gene expression analysis of F1 hybrid mice from CAST x FVB reciprocal crosses showed preferential gene expression from the maternal X compared to paternal X chromosome, revealing that these parent-of-origin effects are not strain specific. SJL mice also showed a parent-of-origin effect on DNA methylation and X gene expression, however which X genes were affected differed from those in C57BL/6. Together, this demonstrates how parent-of-origin differences in DNA methylation of the X chromosome can lead to sex differences in gene expression during immune responses.

2.2 Significance

Sex differences are naturally occurring disease modifiers that, if understood, could lead to novel targets for drug development. Autoimmune diseases are more prevalent in women than men, and sex differences in immune responses have been shown in humans and mice. Here, we discover a global parent-of-origin difference in DNA methylation on the X chromosome that affects gene

expression in activated $CD4^+$ T lymphocytes. The paternal X has more methylation than the maternal X, with higher expression of X genes in XY cells since they only express from the maternal X. Thus, parent-of-origin differences in DNA methylation of X genes can play a role in sex differences in immune responses.

2.3 Introduction

Women have more robust immune responses to self and foreign antigens compared to men. This robust immune response is consistent with a higher incidence of autoimmune diseases in women[1, 2]. In the autoimmune disease multiple sclerosis (MS), women are more susceptible than men by a 3:1 ratio, and in systemic lupus erythematosus (SLE) the female bias is 9:1. This female preponderance across distinct autoimmune diseases suggests a fundamental mechanism underlying its etiology. Female predominate immune responses are also observed across species[1, 3], consistent with the importance of sex as a biological variable[4, 5]. Sex differences can be due to sex hormones, sex chromosomes, or both [6]. While the role of sex hormones has been well studied in preclinical models of autoimmune diseases and in clinical trials, the role of sex chromosomes in autoimmunity remains unclear[6, 7].

Sex chromosomes can cause differences in gene expression between males (XY) and females (XX) due to the expression of Y chromosome genes, X gene dosage effects, or parent-of-origin differences in DNA methylation of X genes[6]. The Y chromosome has evolved from an autosomal ancestor to primarily include genes involved in male reproduction, with only a few non-reproduction related genes remaining[8, 9]. That said, consomic mice previously showed a strain-specific Y chromosome effect on autoimmune disease susceptibility, suggesting that allelic variants of Y genes in a given strain may confer increased disease risk[10, 11].

The X chromosome has many immune related genes and has been widely implicated in sex differences in autoimmunity[3, 12-14]. Females have two X chromosomes while males have one. To

compensate for double expression of X genes, females randomly silence gene expression from one of their X chromosomes by a dosage compensation mechanism called X-inactivation. X-inactivation is initiated by the expression of *Xist* on the inactive X chromosome. *Xist* RNA transcripts associate with the inactive X chromosome to induce gene silencing[15]. While the vast majority of genes on the inactive X are silenced by random X-inactivation, 3% of X genes in mice (15% in humans) escape inactivation[16-18]. That said, the expression level from the inactive X chromosome is typically less than that from the active X[16, 17]. Together, this can lead to X-dosage effects with higher expression of X genes in females (XX) as compared to males (XY).

The third possible mechanism underlying differences in gene expression between XY and XX involves parent-of-origin differences in DNA methylation of X genes. Using the "four core genotypes" model, we previously showed higher expression of the X gene *toll-like receptor 7* (*Tlr7*) in XY cortical neurons compared to XX in experimental autoimmune encephalomyelitis (EAE), a classic $CD4^+$ T lymphocyte mediated model of MS[19]. Higher expression of *Tlr7* in XY as compared to XX cannot be explained by an X dosage effect, since X dosage effects lead to higher expression in XX. It could, however, be due to differences in DNA methylation of X chromosome genes. Inherited differences in DNA methylation that depend on parent-of-origin are often due to epigenetic modifications in the parental germline, namely parental imprinting. Males and females differ in X chromosome origin in that females (XX) inherit both an X chromosome of maternal origin (X_m) and of paternal origin (X_p), while males (XY) inherit only X_m. Random X-inactivation in females inactivates X_m in half of the cells, and X_p in the other half. Thus, females are a mosaic of cells expressing genes from either X_m or X_p[20, 21], whereas males always express genes from X_m. Since DNA methylation typically silences gene expression, parental imprinting of X genes can induce gene expression differences in XX versus XY[22, 23].

The DNA of the inactive X is highly methylated due to X-inactivation[24]. This creates a major confound in investigating possible parent-of-origin differences in DNA methylation patterns when

comparing XX and XY. Studies to date addressing differential DNA methylation based on parent-of-origin have only been done at the transcription level and have not shown direct DNA methylation differences of the X chromosome[25, 26]. Here, in order to study parent-of-origin effects on DNA methylation of the X chromosome without the confound of DNA methylation from X-inactivation, we used a model in which there is only one X chromosome, and therefore no X-inactivation. The one X chromosome was of either maternal (X_m) or paternal (X_p) origin. We used this model to show for the first time a direct parent-of-origin difference in DNA methylation of the X chromosome in autoantigen stimulated $CD4^+$ T lymphocytes.

2.4 Results

Several X genes have higher expression in $CD4^+$ T lymphocytes from XY compared to XX

We previously identified a role for sex chromosomes in modulating the immune response in EAE[12, 27], but whether this was due to Y gene expression, X dosage effects, or parent-of-origin differences in DNA methylation of X genes remained unknown. Here, to investigate transcriptional differences arising from different sex chromosome genotypes in autoimmunity, we analyzed genome wide transcriptomes of autoantigen stimulated $CD4^+$ T lymphocytes from the "four core genotypes" (FCG) mouse model using high throughput RNA sequencing (RNA-Seq). The FCG model utilizes the Y^- chromosome, a Y chromosome with a deletion of the gene responsible for testicular development, namely *sex determining region of the Y* (*Sry*). Thus, XY^- mice are gonadal females. Comparison between XX and XY^- gonadal females permits the study of sex chromosome genes without confounding effects of differences in sex hormones[28, 29]. Transcriptomes of autoantigen stimulated $CD4^+$ T lymphocytes from XX and XY^- mice 10 days after immunization with autoantigen showed separation using principal component analysis (Figure 2-1A), indicating that sex chromosomes altered the transcriptome. The transcriptome data was then used to generate a volcano plot to display differentially expressed genes between XX and XY^-. Two X

genes known to escape X-inactivation, *Kdm6a* and *Kdm5c*[16, 30], had higher expression in XX compared to XY^-, thereby validating sequencing results. Interestingly, we found a cluster of five X genes, *Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7*, which had higher expression in XY^- than XX (Figure 2-1B). This result could not be an effect of X gene dosage which would result in an opposite effect, namely higher expression in XX. Instead, higher expression of an X gene in an XY genotype could be explained by differential DNA methylation of X genes, with more methylation on X_p compared to X_m. A complete list of differentially expressed genes is shown in Dataset S2-1.

We repeated this experiment in gonadal males of the FCG which have the *Sry* transgene inserted at chromosome 3[31, 32], so XX*Sry* and XY^-*Sry* mice both have testes [28, 29]. Comparison between XX*Sry* versus XY^-*Sry* gonadal males also permits the study of sex chromosome genes without confounding effects of differences in sex hormones. Higher expression of the same cluster of five X genes was observed in XY^-*Sry* compared to XX*Sry* (Figure 2-1 C-D). Also, higher expression of *Kdm6a* and *Kdm5c* in XX*Sry* compared to XY^-*Sry* was again observed.

To determine if the observed pattern of X gene expression was related to autoimmune activation, we analyzed the transcriptomes of $CD4^+$ T lymphocytes from nonimmunized, healthy XX and XY^- mice of both gonadal types that were stimulated with anti-CD3 and anti-CD28 antibodies. Consistent with results in $CD4^+$ T lymphocytes from immunized mice, the transcriptomes from $CD4^+$ T lymphocytes from nonimmunized, healthy XX and XY^- mice separated by PCA in both gonadal types (Figure 2-1 E-G) and showed higher expression of the cluster of five X genes in XY^- compared to XX in both sexes (Figure 2-1 F-G). These findings in nonimmunized, healthy mice suggested parent-of-origin imprinting effects in $CD4^+$ T lymphocytes. Notably, higher expression of *Kdm6a* and *Kdm5c* in XX compared to XY^- was again observed in anti-CD3/CD28 stimulated $CD4^+$ T lymphocytes from nonimmunized, healthy mice.

Expression of a cluster of five X genes is higher in CD4$^+$ T lymphocytes from XY compared to XX

To complement our genome wide, unbiased approach, we validated the expression of the five X genes with higher expression in XY$^-$ from our RNA-Seq experiments (*Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7)*. Quantitative RT-PCR analysis of RNA from autoantigen stimulated CD4$^+$ T lymphocytes from two separate sets of immunized mice validated higher expression of all five genes in XY$^-$ compared to XX (Figure 2-2 A-E). *Tlr7* is of particular interest due to its known complex role in immunity [33-40], therefore, we further investigated TLR7 expression at the protein level. Indeed, autoantigen stimulated CD4$^+$ T lymphocytes from XY$^-$ mice had higher TLR7 protein expression than those from XX (Figure 2-2 F-H).

In addition to XX and XY$^-$ gonadal females, we analyzed the expression of the same five X genes in gonadal males of the FCG. We found higher expression of all five X genes in autoantigen stimulated CD4$^+$ T lymphocytes from XY$^-$*Sry* compared to XX*Sry* (Figure 2-2 I-M). TLR7 protein expression was also found to be higher in XY$^-$*Sry* compared to XX*Sry* (Figure 2-2 *N-P*).

We also analyzed TLR7 protein expression in B lymphocytes to determine if differential expression was limited to CD4$^+$ T lymphocytes. We found higher expression of TLR7 in CD19$^+$ B lymphocytes from XY$^-$ and XY$^-$*Sry* compared to XX and XX*Sry*, respectively (Figure S2-1), consistent with findings in CD4$^+$ T lymphocytes (Figure 2-2).

A model to directly study parent-of-origin differences in DNA methylation

To directly investigate potential parent-of-origin differences in DNA methylation of X chromosome genes, we used CD4$^+$ T lymphocytes from X-monosomic mice from the XY* mouse model[32, 41] permitting the comparison of DNA methylation of X_m and X_p without the confound of methylation due to X-inactivation (Figure 2-3). The Y* chromosome is a rearranged Y chromosome with a translocated X chromosome centromere and a modified X pseudoautosomal region (PAR) (Figure 2-3A and 32). When breeding XY* male mice with XX females, the Y*

chromosome undergoes recombination with the X chromosome to produce gonadally female XY*^{x} offspring (Figure 2-3D and 32). The Y*^{x} chromosome is an X chromosome with a major deletion of about 99% of genes, leaving only the PAR and about 8 non-PAR X (NPX) genes (Figure 2-3 A-B and 32). Since XY*^{x} mice are almost completely monosomal for the X chromosome, we refer to them here as "XO" for simplicity. In this genotype, there is no *Xist* expression (Figure 2-3E) and therefore no DNA methylation related to X-inactivation, permitting the direct study of parent-of-origin differences in DNA methylation of X genes.

To determine if differential DNA methylation could be playing a role in differential expression of *Tlr7*, we used CD4^{+} T lymphocytes from this model to analyze the region upstream of the *Tlr7* transcriptional start site (TSS) using targeted bisulfite sequencing. We identified more methylation on X_p than X_m at all four CpG sites analyzed (Figure 2-3F). This result was consistent with higher *Tlr7* RNA expression from X_mY^- versus X_mX_p (Figures 2-1 and 2-2).

The X_p chromosome has significantly more DNA methylation in CpG islands than X_m.

Next, we asked if differential DNA methylation could be occurring on other X genes as it did for *Tlr7*. Thus, we directly analyzed the DNA methylome of CD4^{+} T lymphocytes from X_mO and X_pO mice 12 days after immunization with autoantigen. The X_p chromosome displayed a strikingly higher number of methylated CpG islands in comparison to X_m (Figure 2-4 A-B, Figure S2-3 and Table S2-1, X_p = 45.2%, X_m=1.1%, $p<2.2x10^{-16}$), while no significant difference was observed between X_pO and X_mO autosomes (Figure 2-4 A-B, Figure S2-4 and Table S2-1, p=0.4258). A complete list of differentially methylated genes is shown in Dataset S2-2. The relationship between RNA expression and DNA methylation was examined for the genes which had higher expression in XY$^-$ compared to XX in Figure 2-1 (*Hccs*, *Msl3*, *Prps2*, *Tmsb4x, Tlr7*). All five X genes showed higher DNA methylation on X_p, consistent with the role of DNA methylation in X gene expression (Table 2-1).

Parent-of-origin differences on DNA methylation are not strain specific

To determine whether the effects of DNA methylation are strain specific, we analyzed allele specific gene expression patterns from F1 hybrid mice. By using the F1 generation from CAST/EiJ x FVB/NJ reciprocal crosses, the sequences from X_m or X_p chromosomes can be identified based on SNP differences between these two strains. This permits detection of gene expression differences between X_m and X_p. There was a preference for gene expression from the X_m compared to X_p across several tissues, while very few X genes had higher expression in X_p (Figure 2-4 C-D). Further, we observed that many genes which had higher expression from the X_m in F1 mice were the same genes with more DNA methylation on the X_p (Figure S2-5). Together, this is consistent with parental imprinting as a mechanism for increased expression of X genes in XY mice in more than one strain.

Which genes show differential expression due to parent-of-origin DNA methylation depends on genetic background

Our data indicated that differential parent-of-origin DNA methylation of the X chromosome can occur in several strains, but whether gene expression changes are the same across different genetic backgrounds was not known. To investigate this, we analyzed the transcriptomes of both autoantigen and anti-CD3/CD28 stimulated $CD4^+$ T lymphocytes from XX and XY^- SJL mice of both gonadal sexes (Figure S2-6). The SJL is known to have sex differences in immune responses[42-45]. While SJL mice did not show differential expression of the same five X genes observed in C57BL/6 mice, $CD4^+$ T lymphocytes from XY^- as compared to XX SJL mice had higher expression of *Xlr3b*, an X gene previously reported to be imprinted[46], and this was observed in both immunized and nonimmunized, healthy SJL mice. Thus, which genes show differential expression due to parent-of-origin DNA methylation are not the same across strains. Additionally, there was a *Sry* effect in the SJL, whereby the *Xlr3b* gene did not have higher expression in XY^-*Sry* compared to XX*Sry* gonadal males. A difference in gonadal females versus

males in the FCG model is consistent with previous reports showing an effect of endogenous testosterone in the SJL strain, but not in the C57BL/6 strain in EAE[47]. Notably, *Kdm6a* and *Kdm5c* again had higher expression in XX compared to XY^- regardless of immunization, gonadal type, or strain.

2.5 Discussion

In this study, we performed a genome wide transcriptome analyses in the "four core genotypes" mouse model to determine differences in gene expression due to sex chromosome complement. RNA sequencing analyses of autoantigen stimulated $CD4^+$ T lymphocytes from autoantigen immunized C57BL/6 mice showed a cluster of five X genes with higher expression in XY^- as compared to XX. Quantitative RT-PCR in a separate set of mice confirmed higher expression of *Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7* in XY^- compared to XX. These results were also observed in $CD4^+$ T lymphocytes from nonimmunized, healthy FCG mice stimulated with anti-CD3/CD28 antibodies. Higher expression of an X gene in the XY compared to the XX genotype is in the opposite direction from an X-dosage effect, but is consistent with a parent-of-origin effect on DNA methylation [23]. Examination of the whole methylome in $CD4^+$ T lymphocytes from X-monosomic mice (X_mO and X_pO) showed that the X_p chromosome had a global increase in DNA methylation at CpG islands compared to X_m. CpG islands are regions of DNA with high CG content found near gene promoters and transcriptional start sites that repress gene expression when methylated. The accumulation of DNA methylation at CpG islands throughout the X_p indicated specific silencing of several genes on the paternal X chromosome. Indeed, DNA methylation analysis for differentially expressed genes in $CD4^+$ T lymphocyte transcriptome data showed that X genes with higher expression in XY^- compared to XX had more DNA methylation on X_p.

Our methylome data showing more DNA methylation on X_p than X_m in adult $CD4^+$ T lymphocytes is consistent with known paternally biased DNA methylation during development,

including the preferential inactivation of X_p prior to embryo implantation, thought to be due to imprinting[48-52], and meiotic sex chromosome inactivation during spermatogenesis[53], leading to a more methylated X_p in the germ line. Since DNA methylation patterns from parental imprinting are inherited by all progeny cells, canonically imprinted genes are not tissue specific [54], consistent with our finding of preferential gene expression from X_m across tissues (Fig. 4 *C and D*). Our DNA methylation results are also consistent with previous reports in neonatal brains that showed preferential gene expression from X_m over X_p[25, 26]. To our knowledge, ours is the first study to directly demonstrate parent-of-origin differences in DNA methylation between X_m and X_p without the confound of X-inactivation as a chromosome-wide, rather than a localized[25], effect on the X chromosome. Moreover, these differences in DNA methylation aligned with differential expression of X genes.

Other mechanisms affecting X chromosome gene expression include skewed X-inactivation, whereby X-inactivation is not random, but instead biased toward either the X_m or X_p, and this has previously been suggested as a possible factor in female susceptibility to autoimmune disorders[55, 56], albeit this remains unproven[57]. Skewed X-inactivation is not infrequent in healthy females[58]. Notably, parent-of-origin differences in DNA methylation which drive differences in gene expression from X_m versus X_p are a means by which skewed X-inactivation could alter gene expression. Thus, our findings here of parent-of-origin effects on DNA methylation of the X chromosome support a mechanism of action regarding how skewed X-inactivation could alter gene expression.

Determining why most autoimmune diseases have a higher prevalence in women compared to men is challenging in humans[1, 2]. Since the X chromosome contains many immune related genes, it has been implicated in sex differences in patients with autoimmune diseases[59, 60]. SLE is more prevalent in women than men by 9:1, and men with Klinefelter's syndrome (XXY) have increased susceptibility to SLE as compared to XY men[61, 62]. There have only been 5 reports

of Turner syndrome patients (XO) with SLE, suggesting a lower risk of SLE in XO as compared to XX women[63, 64]. Together, the association of these chromosomal abnormalities with SLE have suggested a possible role of X dosage in autoimmunity, however, studies in humans are confounded by differences in sex hormones in XXY and XO genotypes. In mouse models of SLE, a role of sex chromosomes was shown without the confound of differences in sex hormones. More severe disease and immune dysregulation was demonstrated in XX compared to XY^- mice when the spontaneous lupus susceptible strain (NZM2328) was backcrossed onto the FCG model [13] as well as in pristane-induced lupus when SJL mice were backcrossed onto the FCG model[12].

In multiple sclerosis, the ratio of women to men is 3:1[6], and autoantigen-specific immune responses were shown to be more robust in MS women[65, 66]. Both sex hormones and sex chromosomes have been shown to play a role in sex differences in immune responses during EAE. Previously, draining lymph node cells (LNCs) from MBP immunized SJL mice were stimulated *in vitro* with MBP, then cytokines (TNFα, IFNγ, IL-10) were assessed in supernatants[27]. Wild type (WT) females had higher levels of cytokines than males, and gonadectomy (GDX) suggested a role for both sex hormones and sex chromosomes. An activational effect of adult testosterone to reduce cytokines was shown by an increase in cytokines when males were castrated. A sex chromosome effect was suggested by a difference in castrated males versus ovariectomized females, but this could also be due to an organizational effect of sex hormones prior to GDX. Experiments in the FCG model disentangled these possibilities. There were lower levels of cytokines in gonadally intact males (XX*Sry* and XY^-*Sry*) as compared to females (XX and XY^-), revealing a role for testosterone in decreasing cytokines, either organizational during development or activational during adulthood. The activational effect of testosterone in decreasing cytokines in adults was confirmed by exogenous treatment of adult females with testosterone. Given the major effect of sex hormones, experiments were next done in FCG that were GDX to focus on sex chromosome effects that might be masked by sex hormone effects. Cytokines were

higher in XY$^-$ ovariectomized females (XY$^-$ > XX) and castrated males (XY$^-$*Sry* > XX*Sry*). Together, this was consistent with male sex hormones (testosterone) and male sex chromosome complement (XY) co-evolving to achieve balance[6, 27, 67], with testosterone decreasing cytokines and the XY$^-$ complement increasing cytokines.

Since cytokine changes can have different effects on EAE (TNFα pro-inflammatory, IFNγ variable, and IL-10 anti-inflammatory), the role of sex chromosomes on disease was determined by inducing EAE in SJL FCG mice that were GDX[12]. Adoptive transfer of $PLP_{139-151}$ specific LNCs from XX as compared to XY$^-$ mice showed that XX cells were more encephalitogenic than XY$^-$. When SJL FCG mice were immunized and draining LNCs were restimulated *in vitro* with $PLP_{139-151}$, Th2 cytokines IL-13 and IL-5 were higher in XY$^-$ compared to XX, with similar trends for TNFα, IFNγ, and IL-10. In the current study, we examined genome wide effects, as opposed to hypothesis driven cytokines of interest, in both immunized and nonimmunized, healthy FCG mice on the C57BL/6 and SJL genetic backgrounds. In C57BL/6 mice, XY$^-$ as compared to XX had numerous genes expressed higher in both immunized and nonimmunized mice. This included a cluster of five X chromosome genes which could not be due to X-dosage. Investigation of the methylome provided direct evidence for differences in DNA methylation that aligned with transcriptome data. Notably, in the SJL strain, the same five X chromosome genes were not increased in XY$^-$ mice, but instead there was increased expression of another X gene, one known to undergo parental imprinting (*Xlr3b*) [46]. Another difference between genetic backgrounds (SJL versus C57BL/6) was the role of endogenous testosterone. XX versus XY$^-$ results were the same as XX*Sry* versus XY$^-$*Sry* in the C57BL/6 but differed in the SJL, consistent with a role of endogenous testosterone in EAE in SJL mice[42-45], but not in C57BL/6 mice[47].

A finding shared by the C57BL/6 and SJL strains was higher expression of *Kdm6a* and *Kdm5c* in XX compared to XY$^-$ (and in XX*Sry* compared to XY$^-$*Sry*). These are genes known to escape X-inactivation and are capable of inducing X-dosage effects[16, 30]. *Kdm6a* is a histone

demethylase that regulates expression of other genes, and it showed the greatest increase in XX compared to XY^-. Previously, when *Kdm6a* was selectively deleted in $CD4^+$ T lymphocytes, EAE was ameliorated and the transcriptome showed a decrease in the Neuroinflammatory Signaling Pathway[68]. Thus, an X-dosage effect of *Kdm6a* in females is proinflammatory. While females are more susceptible to EAE in the SJL strain, this is not the case in C57BL/6. We speculate that strain differences in the C57BL/6 and the SJL may be related to the degree of imbalance between higher expression of *Kdm6a* in the XX genotype on the one hand and the five X genes with higher expression in the XY^- genotype on the other. If the five X chromosome genes that are increased in XY^- due to parental imprinting in the C57BL/6 strain have a net proinflammatory effect, then this would balance the proinflammatory effect in XX due to an X-dosage effect of *Kdm6a*. In contrast, the SJL does not have an increase in the five X chromosome genes in XY^-, thereby not balancing the proinflammatory effects in XX due to *Kdm6a.* Clearly the assumption that the net effect of the five X chromosome genes increased in XY^- in the C57BL/6 is pro-inflammatory is highly speculative since *Tmsb4x* and *Tlr7* have complex roles in the immune response[34-40, 69-71] and the remaining genes either involve general functions in apoptosis, differentiation, and proliferation[72, 73], or their role is not yet defined. Regardless of whether this imbalance is ultimately someday shown to contribute to the difference in the sex bias in EAE susceptibility in SJL versus C57BL/6 mice, findings here in the context of existing literature reveal that several factors are involved in sex differences in immune responses. Our overarching hypothesis is that sex differences in the immune system are due to the balance between parental imprinting of X genes that do not escape X-inactivation and X-dosage effects of X genes that do escape X-inactivation, which can be modulated by an effect of sex hormones, as illustrated in Fig. 5.

While our focus here was to study $CD4^+$ T lymphocytes, it is notable that our TLR7 protein expression data in B lymphocytes derived from lymph node cell cultures restimulated with autoantigen was inconsistent with what has been previously described in B lymphocytes from

healthy human and mouse cells[74, 75] and gonadally intact female versus male mice during vaccination[76]. This is likely due to two methodological differences. First, differences in sex hormones in gonadally intact females versus males presents a major confound when studying sex chromosome effects, a confound not present in our studies which used GDX mice of the FCG model. Indeed, many studies have established a role of estrogen on B cells in SLE (humans and mice) [77], and TLR7 function is affected by ERα-ERE interactions[78]. Second, previously observed biallelic expression of *Tlr7* in B lymphocytes was found in cells that were not *in vitro* stimulated[74-76, 79]. Indeed, within 1-2 days of *in vitro* stimulation, most cells had monoallelic expression (70-80% in mice)[74]. Thus, our lymph node cell cultures stimulated for 36 hours *in vitro* would not be expected to show significant biallelic expression of X genes, thereby permitting detection of higher expression of TLR7 in XY^- compared to XX due to parental imprinting.

In summary, our discovery of chromosome-wide hypermethylation of CpG islands on the paternal X chromosome aligns with differential X chromosome gene expression in X_mX_p females compared to X_mY males and provides evidence supporting a novel mechanism involved in sex differences in immune responses.

2.6 Materials and Methods

Mice. SJL/J and C57BL/6J mice were obtained from Jackson Laboratory. MF1 XY^-*Sry* males (Y^- chromosome of 129 origin) were backcrossed with wild type (WT) C57BL/6J or SJL/J females for over 20 generations to obtain litters consisting of the following genotypes: gonadal female XX and XY^- and gonadal male XX*Sry* and XY^-*Sry*. XY* mice on a SJL background were generated by backcrossing B6Ei.LT-Y(IsXPAR;Y)Ei/EiJ males (XY* males, Jackson Laboratory) with wild type SJL/J females for 10 generations. X_mY^{*x} mice were generated by crossing wild type females with XY* males. X_pY^{*x} mice were generated by crossing X_mY^{*x} females with wild type XY males. All animals were allocated into experimental groups based on genotype. Animals were maintained

under standard conditions in a 12-hour dark/light cycle with free access to food and water ad libitum. All procedures were reviewed and approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

Genotyping. XY*^{x} mice were karyotyped using metaphase fibroblasts from ear tissue. Chromosome spreads for at least 8 cells per animal were counted. Only animals that had 39 chromosomes in all cells were used for experiments. XO mice were also genotyped by RT-PCR for *Xist* expression with the following primers: *Xist* (forward: 5'-GTG AGA CGC TTT CCT GAA CC-3', reverse: 5'-ACC CAG TTT TCT GTG CTG CT-3'), *B2m* (forward: 5'- AAT GCT GAA GAA CGG GAA AA-3', reverse: 5'-TGG GGG TGA GAA TTG CTA AG-3'). FCG mice were genotyped by PCR as previously described[31].

Gonadectomy Surgery. To eliminate the effects of circulating sex hormones, female mice (XX, XY^{-}, X_mO, X_pO,) were ovariectomized and male mice (XX*Sry*, XY^{-}*Sry)* were castrated between 4-6 weeks of age. Mice were anesthetized by inhalation of isoflurane. Carprofen (1.2 mg/mL per animal, Zoetis) and 0.5 mL of saline were administered subcutaneously before the procedure. Ovariectomies were performed by removing the fur above the lateral dorsal back using an electric razor and sterilizing the skin with betadine and alcohol scrubs. Bilateral incisions were made into the peritoneum through the back. A hemostat was placed on the fallopian tubes, and the ovaries were removed. The peritoneal muscle layer was closed with absorbable suture (Ethicon) and the skin layer was closed with wound clips. Castrations were performed by removing the fur above the testicular sac using an electric razor and sterilizing the skin with betadine and alcohol scrubs. A central incision into the testicular sac was made. The testicles were located and clamped with the hemostat prior to removal. Following removal of the testicles, the skin was sutured and

secured with a wound clip. For all operated animals, Amoxicillin (0.5 mg/mL, Virbac) was added to the water for 7 days as antibiotic treatment and a second dose of carprofen was given within 24 hours following surgery. Wound clips were removed 7-10 days after surgery. All procedures were approved by the Office of Animal Research Oversight and UCLA institutional animal care and use committee, known locally as the Chancellor's Animal Research Committee.

Immunization. C57BL/6 and SJL/J mice were immunized at 8-12 weeks of age by subcutaneous injection of MOG peptide 35-55 (MOG_{35-55}) (200 ug/mouse, Mimotopes) or PLP peptide 139-151 ($PLP_{139-151}$) (200 ug/mouse, Mimotopes), respectively, emulsified in Complete Freund's adjuvant (BD Bioscience), supplemented with Mycobacterium tuberculosis H37Ra (200 ug/mouse; Difco Laboratories) over draining auxillary and inguinal lymph nodes on Day 0.

Lymphoid Tissue Collection and Lymphocyte Stimulation. Mice were euthanized by a lethal dose of isoflurane. Auxillary, brachial, and inguinal draining lymph nodes were collected and passed through a 40 µm cell strainer to obtain single cell suspensions.

In vitro autoantigen stimulation. C57BL/6 and SJL/J FCG mice were immunized for 10 days. Lymphocytes from single cell suspension were isolated via lymphoprep density gradient (Axis-Shield), washed with 1xPBS, and resuspended in complete RPMI media (cRPMI; RPMI-1640 (Lonza), 5% FBS, 1% L-glutamine (200 mM, Gibco), 1% pen-strep (Gibco), 1% NEAA (100x, Lonza), 1% sodium pyruvate (100 mM, Lonza), 1.25% 1M HEPES (Lonza), and 0.1% 2-merceptoethanol (55 mM, Gibco)). Lymphocytes were plated in 96-well plates at a concentration of $5x10^5$ cells/100 uL with 25 ug/mL of MOG_{35-55} or $PLP_{139-151}$, respectively, plus 20 ng/mL IL-12 and cultured at 37°C with 5% CO_2 for 36 hours. Cells were spun down at 300xg for 10 minutes at 4°C and the supernatants were removed. Cells were harvested and washed with cRPMI media and resuspended in RoboSep Buffer. At least $1x10^6$ cells were saved for flow cytometry studies

in the C57BL/6. The remaining cells were sorted for $CD4^+$ T lymphocytes by negative selection using EasySep Mouse CD4+ T cell isolation kit (STEMCELL) following manufacturer's instructions, homogenized in 600 uL of TriReagent (Zymo Research), and stored at -80°C for RNA isolation.

In vitro anti-CD3/CD28 stimulation. $CD4^+$ T lymphocytes were isolated from single cell suspension of lymph nodes of nonimmunized, healthy C57BL6/J and SJL/J FCG mice by negative selection and cultured in cRPMI medium in 24-well plates at a concentration of 1×10^6 cells/mL with Mouse T-Activator CD3/CD28 Dynabeads (Gibco, Life Technologies) following the manufacturer's instructions at 37°C with 5% CO_2 for 36 hours. Cells were harvested, spun down at 300xg, 4°C for 7 minutes, homogenized in 600 µL TriReagent (Zymo Research), and stored at -80°C until RNA isolation.

RNA Isolation. Chloroform was added to cells suspended in TriReagent (Zymo Research) at a 1:5 ratio and centrifuged for 20 min at 4°C. The aqueous phase was separated for RNA isolation by the Direct-zol RNA microprep kit following manufacturer's instructions (Zymo Research). Final RNA concentrations were measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific).

High Throughput RNA Sequencing and Analysis. RNA sequencing libraries were generated by KAPA Stranded RNA-Seq Kit (Kappa Biosystems) which consists of mRNA enrichment, cDNA generation, end repair, A-tailing, adaptor ligation, strand selection, and PCR amplification. Barcoded adaptors were used for multiplexing samples in one lane. Sequencing was performed on Illumina HiSeq3000 for a single end 1x50 run. Data quality check was done on Illumina SAC. De-multiplexing was performed with Illumina Bcl2fastq2 v2.17 program. These procedures were performed at the UCLA Technology Center for Genomics and Bioinformatics.

Quantitative RT-PCR. cDNA was generated using Tetro cDNA synthesis kit (Bioline). qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) in a Bio-Rad 70 Opticon 2 qPCR/Peltier Thermal Cycler at the UCLA-DOE Biochemistry Instrumentation Core Facility using the following primers: *Mls3* (forward: 5'- ACT TCC ATG CCA GAC CAA CA-3', reverse: 5'-GGT CAA CAT TCT TTT CAG CTG GG-3'), *Prps2* (forward: 5'-GGC TGC GGG GAG ATT AAT GA-3', reverse: 5'- GAG CAC GAC TCT CGC CTA C-3'), *Hccs* (forward: 5'- CGG AAA GGG TGG AAG TGG AA-3', reverse: 5'-CCT CCA AAT CGG ACC AAC GA-3'), *Tmsb4x* (forward: 5'-ATC AGA CTC TCC TCG TTC GC-3', reverse: 5'- TCA ATT GTT TCT TTT GAA GGC AGA G-3'), *Tlr7* (forward: 5'-AAG GGG TAT CAG CAT CTG C AA-3', reverse: 5'-TGC TGA GCT GTA TGC TCT GG-3'), and *Actb* (forward: 5'-GGC TCC TAG CAC CAT GAA GA-3', reverse 5'-ACT CCT GCT TGC TGA TCC AC-3'). *Msl3, Prps2, Hccs, Tmsb4x,* and *Tlr7* Ct values were normalized to *Actb*.

Flow Cytometry. Autoantigen stimulated lymphocytes from C57BL/6J FCG mice were resuspended in FACS buffer (1xPBS + 2% FBS) to a concentration of $1x10^6$ cells/100uL. 16/32 blocking antibody was added at 1:100 dilution and incubated at room temperature for 10 min. Surface proteins were stained with anti-CD4-FITC (Cat# 130308), anti-CD19-APC (Cat# 115512), anti-CD19-PerCP (Cat# 115532), FITC Rat anti-IgG2a,κ (Cat# 400506), APC Rat anti-IgG2a,κ (Cat# 400512), or PerCP Rat anti-IgG2a,κ (Cat# 400530) (1:100, Biolegend) for 20 min at room temperature. The cells were then fixed with 0.5% formaldehyde for 15 min at room temperature and permeabilized overnight in 0.5% saponin at 4°C in the dark. Intracellular proteins were stained with anti-TLR7-PE (Cat# 565557) or PE mouse anti-IgG1,κ (Cat# 550617) (1:200, BD Pharmingen) at room temperature for 30-45 min. Data are representative of two replicate experiments. Flow cytometric analyses were performed on the SORP BD LSRII (IMED) Analytic Flow Cytometer using BD FACSDiva 8.0 and FlowJo (version 10.0.7r2, TreeStar) software.

Positive gates were determined based on isotype controls (Figure S2-2). All flow cytometry was performed at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility.

DNA Isolation. Lymphocyte single cell suspensions from SJL/J XO mice were resuspended in autoMACS Buffer (90 uL/10^7 cells, Miltenyi Biotec) and incubated with anti-CD3 and anti-CD4 microbeads (10 uL/10^7 cells, Miltenyi Biotec) for 15 min at 4°C. The cells were washed with and resuspended in autoMACS buffer (500 uL/10^8cells) then sorted on an autoMACS™ Separator (Miltenyi Biotec) by positive selection. Samples were quick frozen in liquid nitrogen and stored at -80°C until DNA isolation. Genomic DNAs from X_mY^{*x} (X_mO) and X_pY^{*x} (X_pO) $CD4^+$ T lymphocytes were isolated using AllPrep DNA/RNA Mini Kit (Qiagen).

DNA Methylation. Genome-wide DNA methylation was assessed using enhanced reduced representation bisulfite sequencing (ERRBS) as previously described[80]. This method is a modified version of RRBS with significantly improved coverage within and outside of CpG islands, while allowing for accurate quantification of DNA methylation levels at a single base-pair resolution. Briefly, 25ng of genomic DNA was digested with MspI, a restriction enzyme preferentially cutting at CpG rich sites. This digested DNA was purified using phenol:chloroform extraction and ethanol precipitation, before blunt-ending and phosphorylation. A single adenine was added to the 3' end of the fragments in preparation for ligation of methylated and single indexed adapter duplex with a thymine overhang. The ligated fragments were then cleaned and processed for size selection on agarose gel. Selected fragments were treated with bisulfite, followed by PCR amplification to convert unmethylated cytosines to thymine nucleotide. After cleanup with AMPure XP beads, libraries were quantified using the Qubit assay and TapeStation High Sensitivity D1000 kit. Single-end reads of 50 base pairs were obtained for each library by sequencing 3 samples per lane using

HiSeq4000 (Illumina). We then employed FASTQC (version 0.11.3) to assess the overall quality of each sequenced sample and identify specific reads and regions that may benefit from trimming. Next, TrimGalore (version 0.4.0) was employed to trim low-quality bases (quality score lower than 20), adapter sequences (stringency 6) and end-repair bases from the 3' end of reads. For alignment and methylation calling we employed Bismark[81] (version 0.14.3), an integrated alignment and methylation call program that performs unbiased alignment (by converting residual cytosines to thymines prior to alignment in both reads and reference). Briefly, we aligned reads to the reference genome (UCSC mm10 from iGenomes) using Bowtie2[82] (version 2.2.1) with default parameters settings, except for maximum number of mismatches in seed alignment (N) set to 1, and length of seed substrings (L) set to 20. Methylation calls were reported for all nucleotides with a read depth of at least 10. Sites with >500 read depth were removed due to the likelihood that they are biased by duplicates from PCR amplification. We used the methylSig[83] R package (0.4.4) to assess the overall quality of methylation calls and coverage, as well as pairwise differential methylation. For each pairwise comparison methylSig uses a beta-binomial approach to calculate differential methylation statistics, accounting for variation among replicates within each group. We adjusted the p-values for multiple testing using the FDR approach, and considered sites to be differentially methylated when they had a percent change in methylation of at least 15% and a q-value smaller than 0.1. Finally, we annotated sites and regions using UCSC Genome Browser's annotations for CpG islands, promoters and other genetic regions[84]. Enrichment of DNA methylation between chromosomes of $CD4^+$ T lymphocytes from X_mO and X_pO mice was evaluated by Fisher's exact test. Genome wide enhanced reduced representation bisulfite sequencing data that support these findings has been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE122787.

For assessing targeted DNA methylation changes, bisulfite sequencing was performed as previously described[85-88]. Briefly, DNA was treated with sodium bisulfite using the EZ DNA

Methylation kit (Zymo Research, Orange, CA). Sodium bisulfite treatment converts unmethylated cytosine residues to thymine, while methylated cytosine residues remain as cytosines. Sodium bisulfite treated DNA was amplified and directly sequenced (primers available upon request). Percent methylation on each CpG site was quantified using the Epigenetic Sequencing Methylation analysis software (ESME®)[89]. Enrichment of DNA methylation between X chromosomes of $CD4^+$ T lymphocytes from X_mO and X_pO mice was evaluated by Mann Whitney U test.

Allele specific RNA-Seq analysis. RNA-Seq data for F1 hybrid female tissues from reciprocal mating (CAST/EiJ x FVB/NJ and FVB/NJ x CAST/EiJ; maternal allele listed first in each cross) were obtained from GSE75957[90]. A list of single nucleotide polymorphisms (SNPs) was obtained from The Jackson Laboratory (http://www.informatics.jax.org/snp). R package "QuasR" was used for the read alignment to the mouse genome (mm10) in an allele specific way[91], followed by counting at the gene level. In this cross, there is a significant strain effect favoring expression of X genes from the CAST/EiJ X chromosome since FVB/NJ X chromosome alleles are preferentially inactivated. To remove this strain effect, the expression level from CAST/EiJ alleles were divided by the expression level from FVB/NJ alleles after allele specific counting. This (a) normalizes the X_m level from the CAST/EiJ x FVB/NJ cross and (b) normalizes the X_p level from the FVB/NJ x CAST/EiJ cross. The same normalization process was applied for autosomal genes. Since there are only two samples per group, differentially expressed genes were defined such that both samples from one group must be higher (or lower) than the other group.

Statistical Analysis. All statistical analyses were done in a blinded fashion. Statistical analysis and production of figures for RNA-Seq data were performed in R (R Core Team, 2018, http://www.R-project.org/). Qualities of raw sequence data were examined using FastQC (version

0.11.3) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic was used for cleaning[92]. R package "QuasR" was used for the read alignment to the mouse genome (mm10) followed by counting at the gene level[91]. To visualize the relationship of samples, principal component analysis was performed. We assumed read counts followed a negative binomial distribution and constructed generalized linear models based on this negative binomial distribution assumption. Differentially expressed genes between $CD4^+$ T lymphocytes from XX and XY^- mice were identified using R package "edgeR"[93]. A false discovery rate (FDR) of 0.1 was used as the threshold for differentially expressed genes as described[94-97]. See Dataset S1 for a list of differentially expressed genes for all RNA-Seq experiments.

Quantitative RT-PCR and flow data were evaluated by Mann-Whitney U test using GraphPad Prism 8.2.1 for Windows software. The use of the non-parametric Mann Whitney U test was determined based on the F test. Since some analyses required a non-parametric test, we used Mann-Whitney U test for all analyses to make them statistically comparable. Data are representative of two replicate experiments. Data are means ± SD with error bars representing biological variability between mice within each group. Exact P values and group sizes can be found in the figure legends.

Data Availability. Datasets generated during this study are available in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) under the following accession numbers: GSE121292: autoantigen stimulated $CD4^+$ transcriptomes from female C57BL/6J "four core genotypes" mice (10 samples, single end), GSE137793: autoantigen stimulated $CD4^+$ transcriptomes from male C57BL/6J "four core genotypes" mice (10 samples, single end), GSE137791: nonimmunized, healthy anti-CD3/CD28 activated $CD4^+$ transcriptomes from female C57BL/6 "four core genotypes" mice (9 samples, single end), GSE139034: nonimmunized, healthy anti-CD3/CD28 activated $CD4^+$ transcriptomes from male C57BL/6 "four core genotypes"

mice (10 samples, single end), GSE139035: autoantigen stimulated $CD4^+$ transcriptomes from male and female SJL/J “four core genotypes” mice (20 samples, single end), GSE137792: nonimmunized, healthy anti-CD3/CD28 activated $CD4^+$ transcriptomes from male and female SJL/J “four core genotypes” mice (12 samples, single end) and GSE122787: $CD4^+$ DNA methylome from SJL/J XY^{*X} mice (9 samples, single end).

	RNA expression			DNA methylation				
	log_2FC (XY/XX)	*P* value	FDR	methDiff ($X_m - X_p$)	SD	DM CpGs/all CpG	q-value (minimum)	q-value (maximum)
Hccs	1.06	1.18E-11	1.19E-08	−25.6	4.4	11/14	0.0001	0.1590
Msl3	1.09	2.44E-20	3.11E-17	−30.3	2.7	6/7	0.0024	0.1230
Prps2	0.97	5.44E-18	6.36E-15	−41.4	8.5	5/7	0.0263	0.1410
*Tlr7**	1.19	1.87E-07	1.25E-04	−38.0	4.9	4/4	0.0079**	0.0119**
Tmsb4x	1.02	3.80E-10	3.33E-07	−46.1	9.4	4/6	0.0166	0.1890

*measured by targeted DNA methylation analysis
**p-values

Table 2-1. X genes with higher expression in XY have more DNA methylation on X_p compared to X_m. The difference in DNA methylation between X_m and X_p was analyzed for the cluster of five X genes that had higher RNA expression in XY^- compared to XX in autoantigen stimulated $CD4^+$ T lymphocyte transcriptome data from C57BL/6 mice (*Hccs*, *Msl3*, *Prps2*, *Tmsb4x*, *Tlr7*; Figure. 1) to investigate the relationship between RNA expression and DNA methylation. All five genes had more DNA methylation on X_p. FDR<0.1 was used as the threshold for significance for differential RNA expression (R package edgeR). DNA methylation differences for all CpG sites in each gene were averaged to generate one value of differential methylation (DM). q-value<0.1 was used as a threshold for significance of differentially methylated CpG sties (R package methylSig). The q-values for the most significant (min) and least significant (max) CpG site are listed. *Tlr7* DNA methylation data was obtained from targeted DNA methylation analysis and p-value<0.05 (Mann Whitney U test) was used as a threshold for significance of differential methylation.

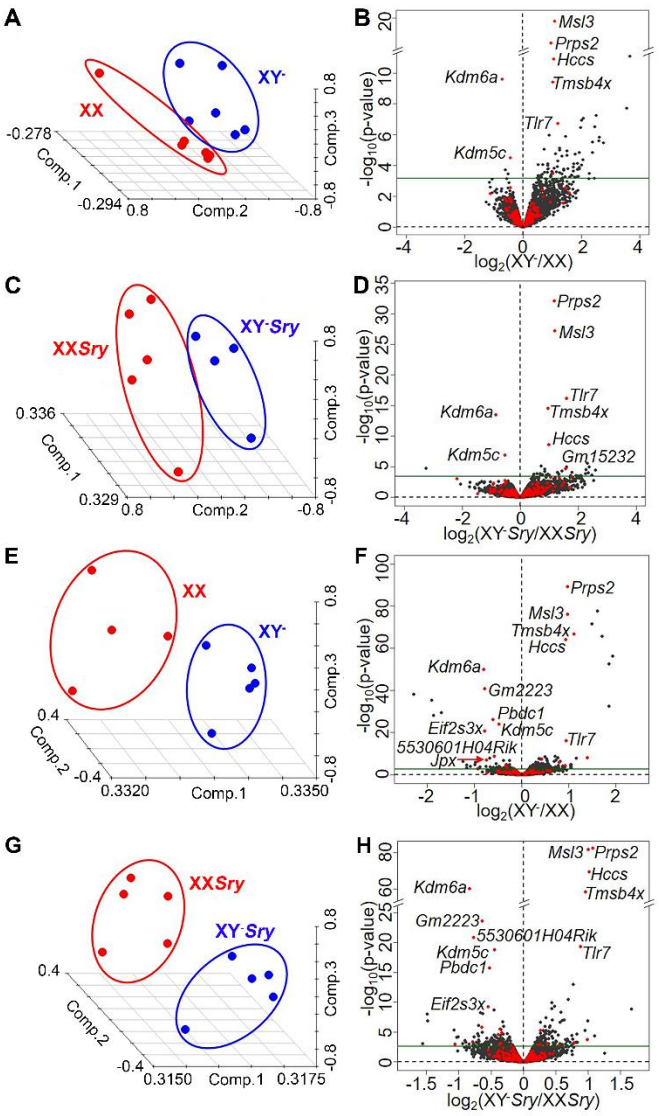

Figure 2-1. CD4⁺ T lymphocytes from XX and XY⁻ mice have distinct transcriptomes. RNA from autoantigen (*A-D*) and anti-CD3/CD28 (*E-H*) stimulated CD4⁺ T lymphocytes from C57BL/6 "four core genotypes" mice was analyzed by RNA-Seq. (*A*) Principle component analysis (PCA) of autoantigen stimulated CD4⁺ T lymphocyte transcriptomes showed separation of XX (n=6) and XY⁻ (n=6). (*B*) Volcano plots showed the distribution of differentially expressed autosomal (black) and X (red) genes between XX and XY⁻. A cluster of five X genes had higher expression in XY⁻ than XX. (*C*) PCA of autoantigen stimulated CD4⁺ T lymphocyte transcriptomes showed separation of XX*Sry* (n=5) and XY⁻*Sry* (n=5). (*D*) Volcano plot showed higher expression of the

five X gene cluster in XY$^-$*Sry* compared to XX*Sry*. (*E*) PCA of anti-CD3/CD28 stimulated CD4$^+$ T lymphocyte transcriptomes showed separation between XX (n=4) versus XY$^-$ (n=5) (*F*) Volcano plot showed higher expression of the five X gene cluster in XY$^-$ than XX. (*G*) PCA of anti-CD3/CD28 stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX*Sry* (n=5) and XY$^-$*Sry* (n=5). (*H*) Volcano plot showed higher expression of the five X gene cluster in XY$^-$*Sry* compared to XX*Sry*. FDR<0.1 was used as the threshold for significance (green line): any gene above this line was considered significantly different (FDR was calculated using R package edgeR).

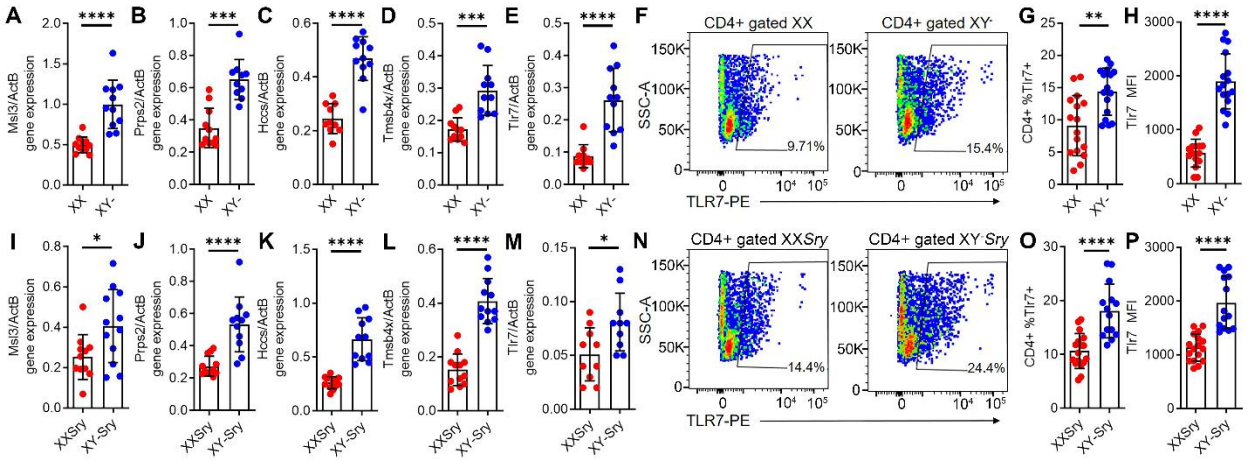

Figure 2-2. XY$^-$ has higher expression of a cluster of five X genes compared to XX. RNA and protein expression were measured in autoantigen stimulated CD4$^+$ T lymphocytes from XX and XY$^-$ mice immunized with autoantigen. (*A-E*) RNA expression of (*A*) *Msl3*, (*B*) *Prps2*, (*C*) *Hccs*, (*D*) *Tmsb4x*, and (*E*) *Tlr7* was measured by quantitative RT-PCR. XY$^-$ (n=10-11) had higher RNA expression than XX (n=10-11) for all five genes (****: p<0.0001, ***: p<0.0002). (*F-H*) CD4$^+$ T lymphocytes were analyzed for TLR7 protein expression by flow cytometry. See *SI Appendix*, Fig. S2 for gating strategy. (*F*) Representative flow plots for TLR7 expression in XX versus XY$^-$. (*G*) XY$^-$ mice (n=16) had a higher percentage of TLR7 expressing CD4$^+$ T lymphocytes than XX (n=16) (**: p=0.0017). (*G*) XY$^-$ mice (n=16) had higher mean fluorescence intensity (MFI) of TLR7 expression in CD4$^+$ T lymphocytes than XX (n=16) (****: p<0.0001). (*I-P*) The same analyses in *A-H* were performed in XX*Sry* and XY$^-$*Sry* gonadal males. (*I*) *Msl3*, (*J*) *Prps2*, (*K*) *Hccs*, (*L*) *Tmsb4x*, and (*M*) *Tlr7* RNA expression; XX*Sry* n=10-12, XY$^-$*Sry* n=10-12 (*: p<0.045, ****: p<0.0001). (*N*) Representative flow plots for TLR7 expression in XX*Sry* versus XY$^-$*Sry*. (*O*) TLR7 percent expression; XX*Sry* n=17, XY$^-$*Sry* n=14 (****: p<0.0001). (*P*) TLR7 MFI; XX*Sry* n=17, XY$^-$*Sry* n=14 (****: p<0.0001). All data are representative of 2 replicate experiments. Error bars represent SD. P values were calculated by Mann Whitney U test.

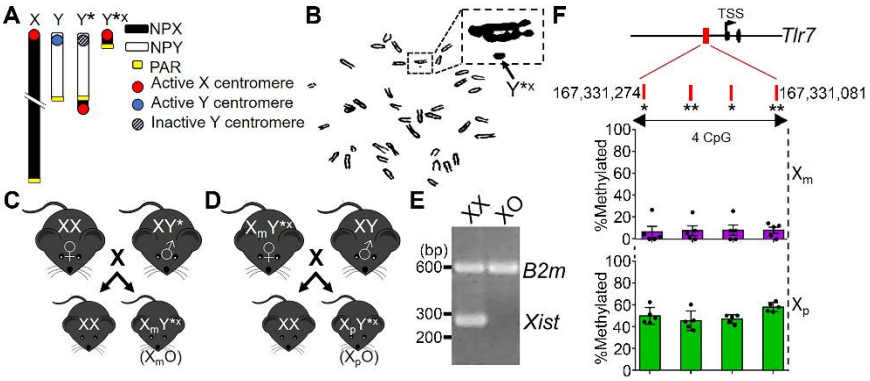

Figure 2-3. A monosomic X mouse model to directly study parent-of-origin differences in DNA methylation. (*A*) Diagrams of the sex chromosomes used in the breeding of XY*^{x} (XO) mice. The Y* chromosome is a rearranged Y chromosome with a translocated X chromosome centromere and a modified X pseudoautosomal region (PAR) (see 32 for details). The Y*^{x} chromosome is produced from recombination of the X and Y* chromosomes. It is an X chromosome with a massive deletion of about 99% of genes, leaving the pseudoautosomal region (PAR) and about 8 non-PAR genes [32]. (*B*) Representative metaphase spread of XY*^{x} (XO) chromosomes. An XY*^{x} animal has 39 normal chromosomes, plus one small "spec" representing the Y*^{x} chromosome (inset). (*C*) Female X_mO mice were generated by crossing wild type XX females and XY* males. (*D*) Female X_pO mice were generated by crossing X_mO females with wild type XY males. (*E*) *Xist* expresses in XX, but not in XO. RT-PCR was performed on cDNA from ear tissue. The expression of *B2m* was used as the internal control. (*F*) DNA methylation of *Tlr7* was analyzed using targeted bisulfite sequencing in $CD4^+$ T lymphocytes of X_mO (n=5) and X_pO (n=5) SJL mice 12 days after immunization with autoantigen. The DNA methylation on X_m and X_p is shown as the percentages of methylation at CpG sites of a CpG island upstream of the *Tlr7* transcriptional start site (TSS). X_p had more methylation than X_m at each site analyzed (*: $p<0.0120$, **: $p<0.0008$, Mann Whitney U test). Error bars represent SEM.

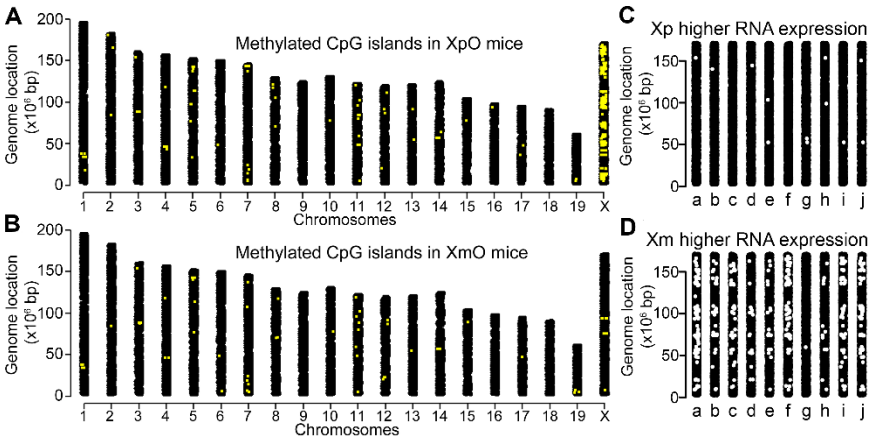

Figure 2-4. Genes on the X_p have more DNA methylation in CpG islands than X_m. X_mO and X_pO female mice were immunized with autoantigen for 12 days. DNA from $CD4^+$ T lymphocytes isolated from lymph nodes was analyzed by bisulfite sequencing to generate the whole methylome of X_pO (n=4) and X_mO (n= 5) SJL mice. Yellow spots indicate CpG sites in CpG islands that had DNA methylation in >50% of reads on average. Black dots were mapped based on all gene locations from the Ensemble genome browser (http://www.ensembl.org) and form the chromosome shapes. (*A*) The X_p chromosome showed greater accumulation of CpG island DNA methylation compared to autosomes ($p<2.2x10^{-16}$, Fisher's exact test). (*B*) The X_m chromosome did not have accumulation of DNA methylation compared to autosomes (p = 1, Fisher's exact test). Comparing X_p (*A*) and X_m (*B*), the number of methylated CpG islands was significantly higher on X_p than X_m ($p<2.2x10^{-16}$, Fisher's exact test). No difference in DNA methylation was observed for autosomal genes between X_pO and X_mO (p=0.4258, Fisher's exact test). (*C and D*) Gene expression data from F1 hybrid mice derived from CAST/EiJ x FVB/NJ and FVB/NJ x CAST/EiJ reciprocal crosses was analyzed to show parent-of-origin differences in X gene expression in multiple tissues (a: E16.5 liver, b: E16.5 brain, c: E16.5 heart, d: day 3 tongue, e: day 3 brain, f: adult brain, g: adult liver, h: adult heart, i: adult lung, j: adult spleen)**.** White dots represent genes on the X chromosome with higher expression from X_p (*C*) or from X_m (*D*). There were many more X genes with higher expression from X_m compared to X_p across tissues.

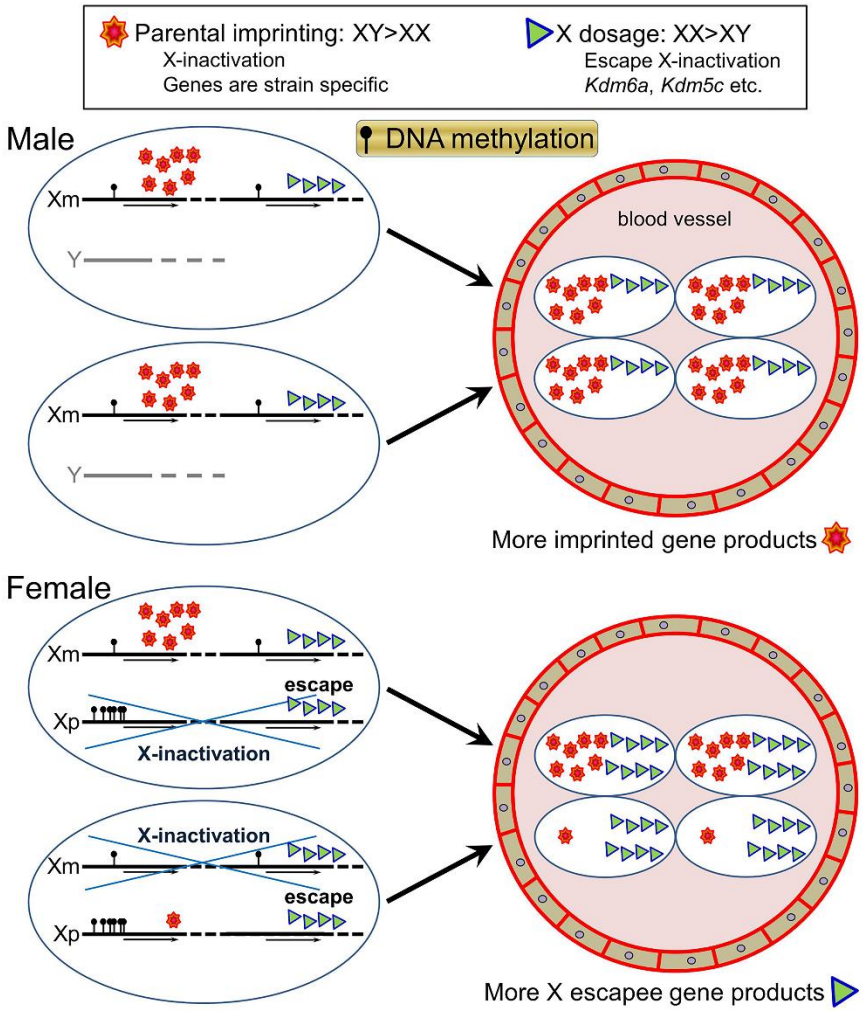

Figure 2-5. Overarching hypothesis that sex differences in the immune system are due to the balance between parental imprinting of X genes that do not escape X-inactivation (red stars) and X-dosage effects of X genes that do escape X-inactivation (blue triangles), which can be modulated by an effect of sex hormones. Males (XY) have one X chromosome with maternal imprinting (higher expression). On the other hand, females (XX) have two X chromosomes, one with maternal and one with paternal imprinting. Due to random X inactivation in females, the X_m is inactivated in half of the cells, while X_p is inactivated in the other half, creating a mosaic of cells expressing genes from either X_m (higher expression) or X_p (lower expression). Additionally, some genes escape X inactivation (*Kdm6a* for example), expressing from both X chromosomes and

creating an X-dosage effect with higher expression in XX versus XY. Together, this results in higher expression of parentally imprinted genes in XY and higher expression of X escapee genes in XX. These sex chromosome complement effects are not mutually exclusive of sex hormone effects, both organizational effects during development and activational effects during adulthood.

	Chr	Genes with methylated CpG (>50%)	Analyzed genes	Percentage (>50%)
X_pO	A	58	446	13.0
	X	122	270	45.2
X_mO	A	49	446	11.0
	X	3	270	1.1

Table S2-2. Percentage of genes with CpG site methylation. A total of 446 autosomal genes and 270 X genes were analyzed for the methylation status of CpG islands. X_p had 122 (45.2%) X genes over 50% methylated, whereas X_m had only 3 genes (1.1%). Autosomal genes in both X_pO and X_mO had approximately 12% genes methylated.

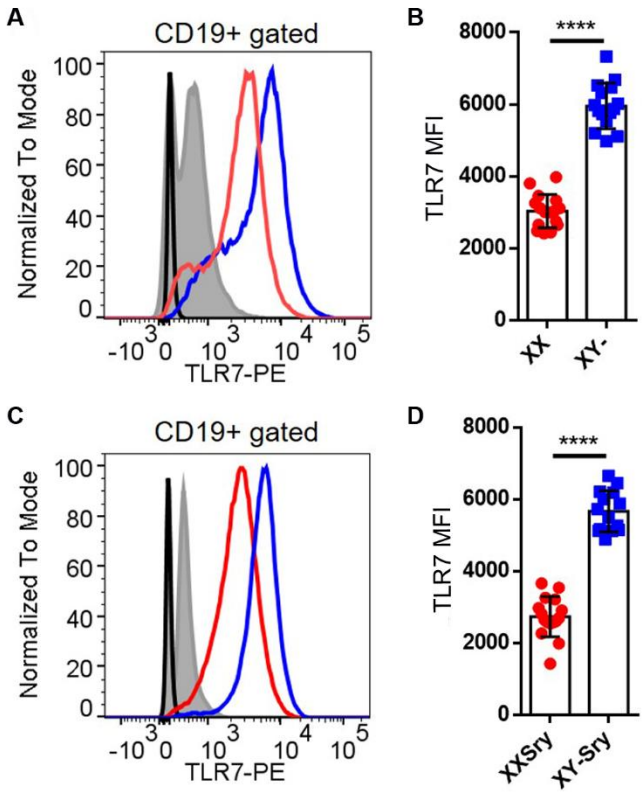

Figure S2-1. B cells from XY$^-$ mice have higher expression of TLR7 compared to XX. (*A*) Autoantigen stimulated lymphocytes from XX and XY$^-$ mice were gated on CD19$^+$ B cells and analyzed for TLR7 protein expression by flow cytometry (black=negative, grey=isotype, red=XX, blue=XY$^-$). (*B*) Mean fluorescence intensities of TLR7 in CD19$^+$ B lymphocytes were graphed. XY$^-$ (n=16) had higher TLR7 expression than XX (n=16) (****: p<0.0001, unpaired, two tailed, t test). Data represent 2 replicate experiments. Error bars represent SD. (*C-D*) The same analyses in *A-B* were performed in cells from XX*Sry* and XY$^-$*Sry* mice. (*D*) XX*Sry* n=16, XY$^-$*Sry* n=16 (****: p<0.0001). Data represent 2 replicate experiments. Error bars represent SD. P values were calculated using Mann Whitney U test.

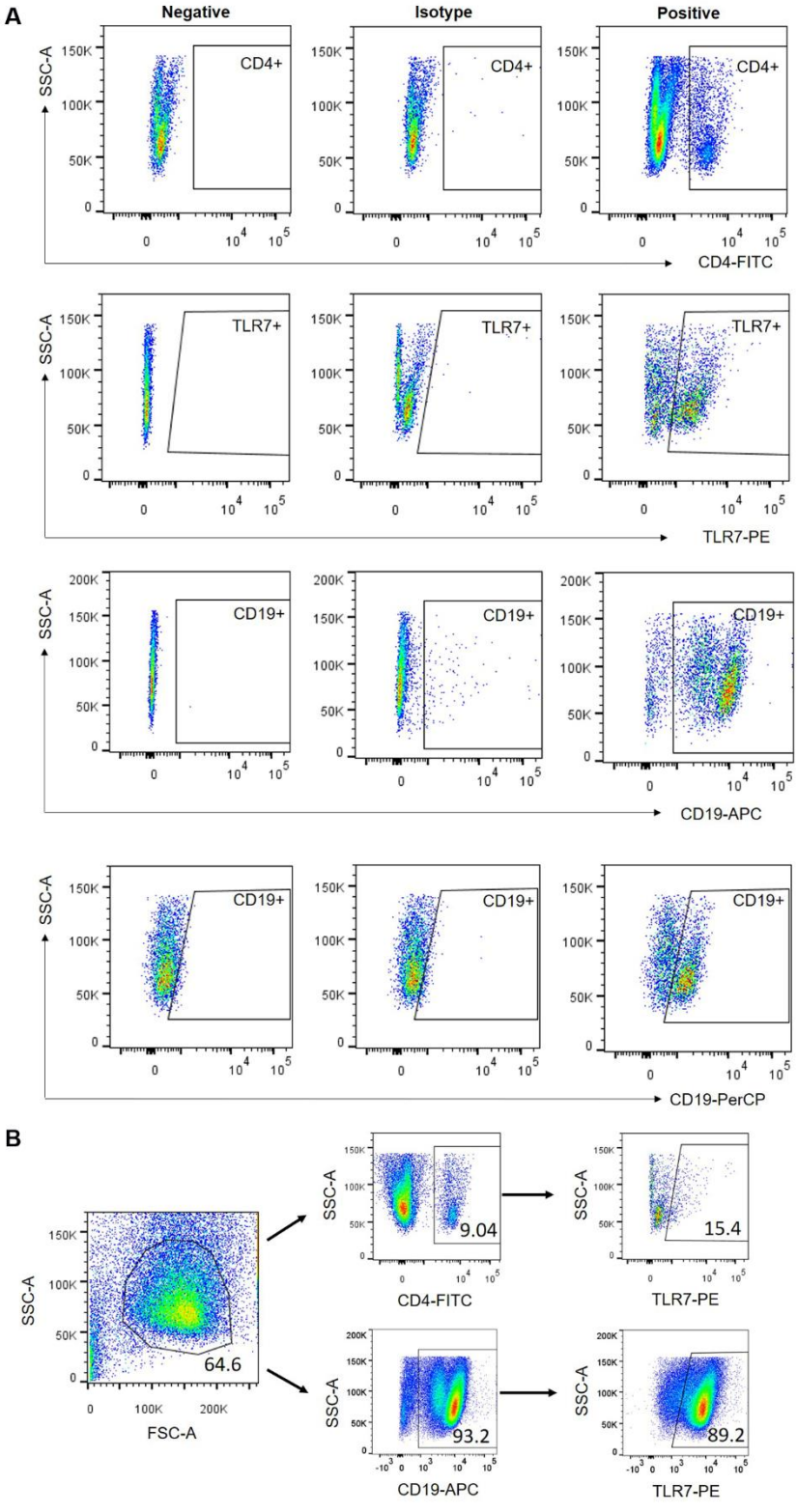
A
Negative
Isotype
Positive
SSC-A
CD4+
CD4-FITC
TLR7+
TLR7-PE
CD19+
CD19-APC
CD19-PerCP
B
FSC-A
64.6
9.04
15.4
93.2
89.2

Figure S2-2. Flow cytometry gating strategy for TLR7 expression. (*A*) Negative, isotype, and positive controls for each stain were evaluated on a mixture of cells from experimental samples. Positive gates were selected based on the isotype controls. (*B*) Gating strategy to measure TLR7 expression in $CD4^+$ T lymphocytes and $CD19^+$ B lymphocytes. Lymphocytes were selected by SSC-A and FSC-A light scattering. Cells were first gated on $CD4^+$ or $CD19^+$ and subsequently gated for $TLR7^+$.

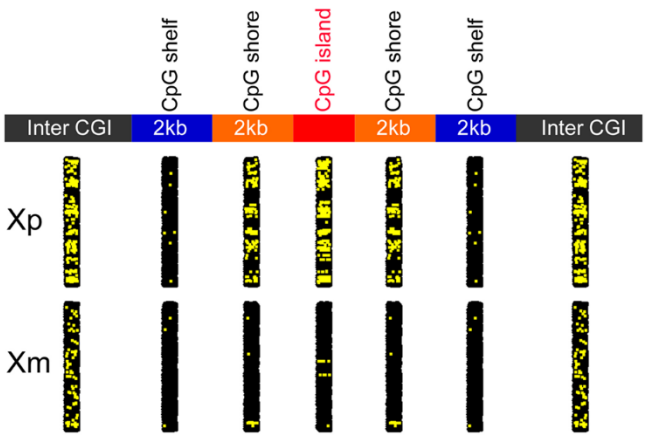

Figure S2-3. CpG island and shore regions on the X_p chromosome are more methylated than on the X_m chromosome. DNA methylation of $CD4^+$ T lymphocytes from X_mO and X_pO immunized mice was analyzed by bisulfite sequencing. CpG islands (CGI) and their surrounding regions for each gene on the X chromosome were mapped based on the CpG annotation in the mm10 mouse genome. Yellow spots indicate CpG sites that have DNA methylation of >50% of reads on average. Black dots were mapped based on all gene locations in the Ensemble genome browser (http://www.ensembl.org) and form the chromosome shapes. The schematic describes the relative genomic location of each CpG category. There were significant differences of X chromosome CpG methylation between X_p and X_m in CpG island regions ($p<2.2x10-16$, Fisher's Exact test), shore regions ($p=2.2x10-15$, Fisher's Exact test), and Inter CGI regions ($p=1.9x10-5$, Fisher's exact test). There was no significant difference in the CpG shelf region ($p=0.3430$, Fisher's exact test).

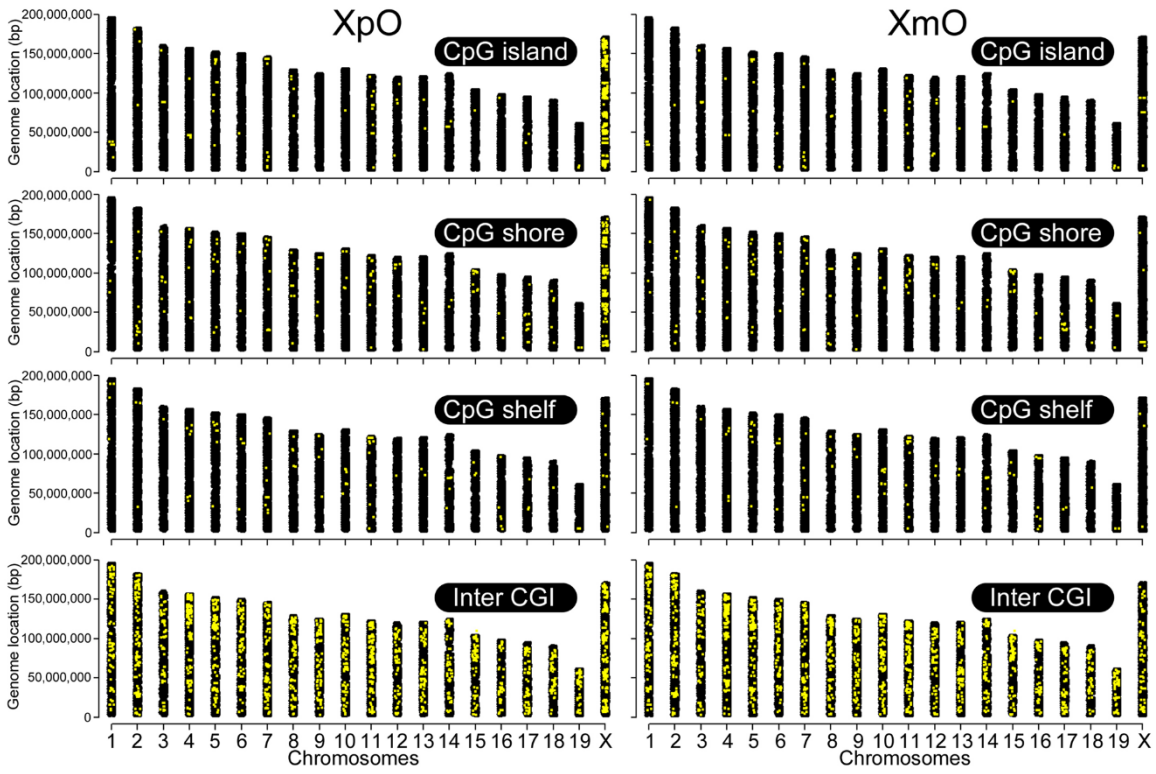

Figure S2-4. CpG methylation is not different between autosomes of X_pO and X_mO. DNA methylation of $CD4^+$ T lymphocytes from X_mO and X_pO immunized mice was analyzed by bisulfite sequencing. CpG islands (CGI) and their surrounding regions for each gene on the X chromosome were mapped based on the CpG annotation in the mm10 mouse genome. Yellow spots indicate CpG sites that have DNA methylation of >50% of reads on average. Black dots were mapped based on all gene locations in the Ensemble genome browser (http://www.ensembl.org) and form the chromosome shapes. The DNA methylation of X_pO and X_mO chromosomes were compared for each CpG category: CpG island, CpG shore, CpG shelf, and Inter CGI. There was no significant difference of autosomal CpG methylation between X_pO and X_mO in any region (CpG island p=0.4258, CpG shore p= 0.5557, CpG shelf p=1.000, Inter CGI p=0.4200, Fisher's exact test).

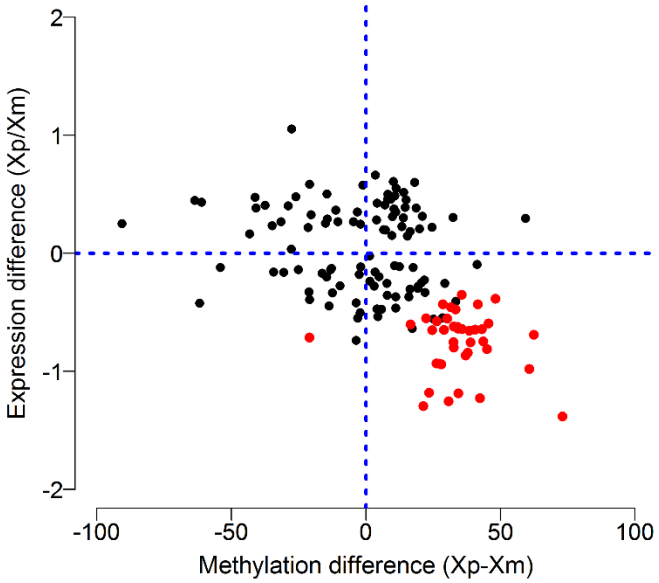

Figure S2-5. Genes expressed higher from X_m compared to Xp have more DNA methylation on X_p compared to X_m. Scatterplot shows the comparison of X_m/X_p gene expression ratios from the spleen of F1 hybrid mice (y-axis) and X_m-X_p DNA methylation differences from SJL $CD4^+$ DNA methylation data (x-axis). Autosomal genes are shown in black, while X genes are shown in red. The distribution of A genes does not show meaningful bias, but X genes localize in the bottom-right quadrant, indicating that X genes are expressed more from X_m and have more DNA methylation on X_p. This suggests that the higher DNA methylation on X_p is suppressing gene expression from X_p,

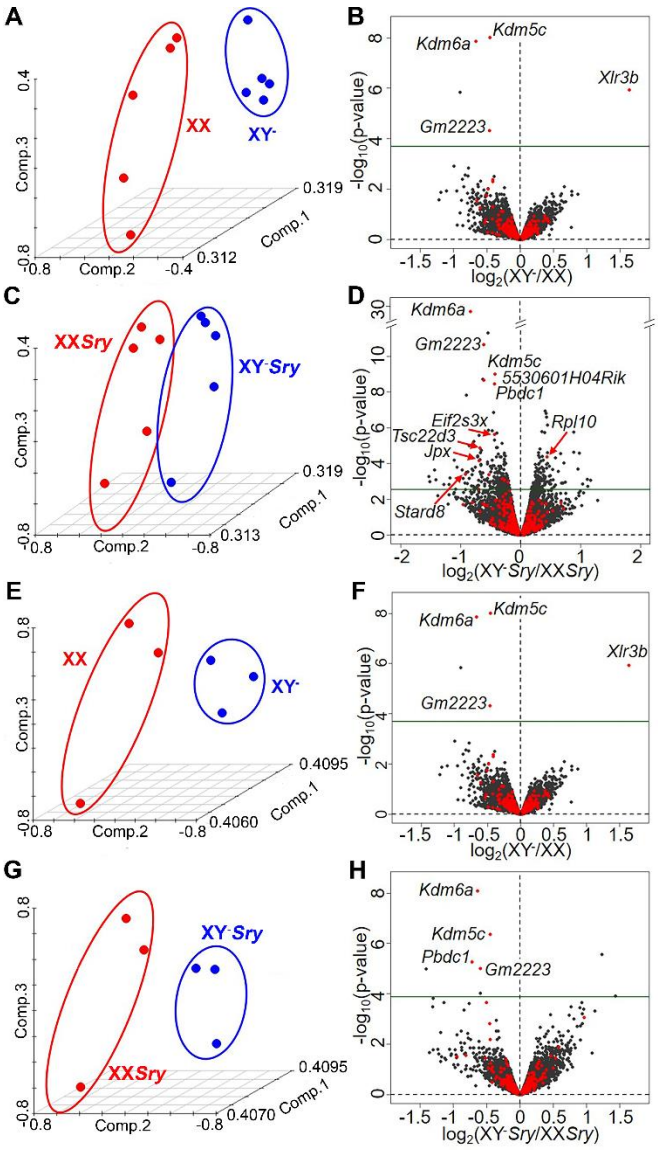

Figure S2-6. CD4$^+$ T lymphocytes from XX and XY$^-$ SJL mice have distinct transcriptomes. RNA from autoantigen (A-D) and anti-CD3/CD28 (E-H) stimulated CD4$^+$ T lymphocytes from SJL "four core genotypes" mice was analyzed by RNA-Seq. (A) PCA of autoantigen stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX (n=5) and XY$^-$ (n=5) (B) Volcano plot showing the distribution of differentially expressed autosomal (black) and X (red) genes between XX and XY$^-$. The X gene *Xlr3b* has higher expression in XY$^-$ compared to XX. (C) PCA of autoantigen stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX*Sry* (n=5) and XY$^-$*Sry* (n=5). (*D*) Volcano plot showed the X gene *Rpl10* had higher expression in XY$^-$*Sry*.

(*E*) PCA of anti-CD3/CD28 stimulated $CD4^+$ T lymphocyte transcriptomes showed separation between XX (n=3) versus XY^- (n=3). (*F*) Volcano plot showed the X gene *Xlr3b* has higher expression in XY^- compared to XX. (*G*) PCA of anti-CD3/CD28 stimulated $CD4^+$ T lymphocyte transcriptomes showed separation between XX*Sry* (n=3) and XY^-*Sry* (n=3) (*H*) Volcano plot showed no X genes had higher expression in XY^-*Sry*.

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Chapter 3

The Role of Hippocampal Astrocytes in Neurodegeneration During EAE

3.1 Abstract

Current treatments for multiple sclerosis (MS) are immunomodulatory with no direct effects on neurodegeneration. Understanding mechanisms of neurodegeneration in a cell-specific and region-specific way may reveal new targets for neurodegenerative intervention. Astrocytes are key players in the neuroinflammatory response to injury, and have important roles in the pathogenesis of MS. Here, we take a gene expression approach to investigate mechanisms of hippocampal astrocytes that underlie cognitive disability progression using the animal model for MS, experimental autoimmune encephalomyelitis (EAE). We analyzed genome-wide transcriptomes of hippocampal astrocytes during EAE using RiboTag technology. RNA-Seq analysis of astrocyte derived RNA revealed that genes involved in interferon signaling and antigen presentation were upregulated in EAE. Interestingly, the majority of genes in these pathways were related specifically to MHC class I (MHC I) signaling. MHC I has an important role in synaptic plasticity, and has been shown to be expressed in astrocytes under inflammatory conditions. Thus, we hypothesize that MHC I related genes signal to neurons and microglia to induce aberrant synaptic pruning in adult mice with neurodegenerative disease (EAE). Future studies using conditional knockout of the MHC I cofactor B2M in astrocytes will be used to investigate the role of astrocyte MHC I signaling in neurodegeneration. Additionally, MHC I gene expression has been shown to be inversely correlated with estrogen signaling. We will investigate how sex hormones affect MHC I gene expression in astrocytes during EAE in both males and females. These results are the first to suggest a role for astrocyte derived MHC I signaling in EAE that may provide a direct mechanism to therapeutically target neurodegeneration.

3.2 Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by astrogliosis and demyelination, with axonal and synaptic loss. MS causes a variety of symptoms such as cognitive impairment, motor dysfunction, vision impairment, and fatigue, each corresponding to damage in specific regions on the CNS[1]. As such, each MS patient will have different combinations and degrees of symptoms depending on the location, number, and size of immune lesions in the CNS. The distinct role of inflammatory lesions in MS pathology provides a unique opportunity to understand mechanisms of neuroinflammation that may also be relevant to other neurodegenerative diseases such as Parkinson's and Alzheimer's disease which also have a neuroinflammatory component[2, 3]. Current available treatments for MS are immunomodulatory, reducing relapses by one-half to one-third, with only mild and indirect effects on neurodegeneration. No treatments exist that directly target neurodegeneration[4]. An in-depth understanding of neurodegenerative mechanisms will aid in developing therapeutics to prevent or reverse neurodegeneration.

One approach to understanding these mechanisms is to investigate gene expression changes at the RNA level. Studying gene expression in the CNS during neuroinflammatory disease is complex since changes in whole tissue gene expression reflect not only gene expression changes in different cells, but also changes in cell composition due to proliferation, migration, and cell loss during disease. Analyzing whole tissue can overlook important genes and pathways in individual cell types[5]. Therefore, it is important to investigate changes in gene expression in a cell-type-specific manner. Astrocytes play pivotal roles in MS pathology; they create the multiple sclerotic lesions in the CNS that give MS its name[6]. In response to neuroinflammation, astrocytes become reactive and have complex roles depending on the microenvironment and timing of disease[7]. They exhibit deleterious roles via several avenues: activating the innate immune response, releasing pro-inflammatory cytokines and chemokines,

and preventing remyelination and axonal regeneration. However, astrocytes may also be protective by: preventing the spread of neurodegeneration and infiltrating cells outside the lesion, releasing anti-inflammatory cytokines and chemokines, promoting oligodendrocyte precursor cell maturation, transporting glutamate out of synapses, and secreting neurotrophic factors [7-9]. Astrocytes are protective and help attenuate disease in the early phase of the preclinical model for MS, experimental autoimmune encephalomyelitis (EAE). However, in the late progressive phase of EAE, astrocytes are deleterious and exacerbate disease[10]. Additionally, astrocyte specific deletions of pro-inflammatory genes NFκB and CCL2 provide protection against progression and demyelination in EAE[11, 12]. We recently showed that astrocytes downregulated cholesterol biosynthesis in the spinal cord during EAE, and treatment to restore cholesterol homeostasis ameliorated disease[5]. Thus, understanding key mechanisms of the astrocytes response to injury and inflammation may provide key insights and targets for neurodegenerative disease.

Astrocytes have heterogeneous phenotypes within the healthy CNS[13, 14] and in response to injury[15]. Specifically, we showed that astrocytes in different CNS regions have distinct gene expression profiles during EAE[5]. Therefore, analyzing gene expression changes in astrocytes in specific regions of the CNS is critical to discovering disability specific mechanisms of neurodegeneration. Over 50% of MS patients experience cognitive impairment, including hippocampal dependent memory loss[16-21], stressing the importance of understanding mechanisms of neurodegeneration in the hippocampus.

Here, we took a gene expression approach to understand mechanisms of astrocytes in the hippocampus during the late chronic phase of EAE. We used RiboTag technology to isolate astrocyte specific mRNA from the hippocampus of normal and EAE mice and analyzed the translatomes by high throughput RNA sequencing (RNA-Seq). MHC class I (MHC I) related genes and pathways were upregulated in hippocampal astrocytes during EAE compared to normal

healthy controls. These results were validated by quantitative RT-PCR (qRT-PCR) and immunohistochemistry. Future studies will investigate how astrocyte MHC I affects synaptic loss during disease and how sex hormones affect the expression of MHC class I signaling genes in astrocytes.

3.3 Results

Specificity of astrocyte mRNA isolation from GFAP-Cre(77.6)xRiboTag mice.

In order to isolate RNA specifically from astrocytes, we used RiboTag technology[22]. RiboTag utilizes the Cre-Lox system to add a hemagglutinin (HA) tag to ribosomal subunit RPL22 which can be used for HA-mediated immunoprecipitation of ribosomes and associated mRNAs. Here, *Cre* expression is driven by the astrocyte-specific mGFAP(77.6)-Cre promoter, thereby enabling the HA tag to be expressed on ribosomes of astrocytes. Importantly, this technology allows the cells of interest to remain in their three-dimensional conformation with other cell types until RNA isolation. To confirm that the HA tag is expressed specifically in astrocytes in the hippocampus, we analyzed the colocalization of HA protein expression with cell-type specific markers using immunohistochemistry (Figure 3-1 A-C). HA colocalized strongly with astrocytes (GFAP, ALDH1L1) (Figure 3-1A), while there was little to no colocalization with neurons (NEUN), microglia (IBA1, P2Y12), or oligodendrocytes (CC1) (Figure 3-1 B-C).

We next validated the cell specificity of the immunoprecipitation technique. qRT-PCR analysis of mRNAs isolated from the hippocampus validated enrichment of astrocyte genes (*Gfap*, *Aldh1l1*) and deenrichment of neuronal (*Syp*, *Calb1*), microglial (*Tmem119*), and oligodendrocyte (*Mbp*) genes in the HA immunoprecipitated fraction compared to flow through (Figure 3-1D).

EAE alters gene expression in hippocampal astrocytes.

After validating astrocyte specificity of the RiboTag technique, we analyzed mRNAs from hippocampal astrocytes of EAE day 45 mice as well as normal age matched controls by RNA-Seq. There were 208 differentially expressed genes between EAE and normal hippocampal astrocytes driving the separation of the groups by principle component analysis (PCA) (Figure 3-2A). The majority of the significantly differentially expressed genes were upregulated during EAE (Figure 3-2B). Canonical pathway analysis of the differentially expressed genes between EAE and normal astrocytes identified Interferon Signaling and Antigen Presentation Pathway as the top two differentially regulated pathways. The expression of all genes included in both pathways was strongly upregulated during EAE (Figure 3-2D). Interestingly, 11/14 of the genes associated with these pathways are specifically related to MHC class I signaling.

MHC class I signaling is increased in hippocampal astrocytes during EAE.

The results from our RNA-Seq analysis were validated by qRT-PCR analysis of five MHC class I related genes (*Tap1, Tap2, Stat1, Psmb8, Isg15*) from the above pathways on RNA samples from two separate cohorts of male normal and EAE mice (Figure 3-3). All five genes had significantly higher expression in EAE compared to normal astrocytes (Figure 3-3A). Interestingly, MHC I expression has been shown to negatively correlate estrogen signaling. Therefore, we wanted to know if these five genes were also differentially expressed in hippocampal astrocytes from females. Indeed, all 5 genes were upregulated in EAE compared to normal females (Figure 3-3B).

The upregulation of TAP1 in astrocytes during EAE was further investigated at the protein level by analyzing the percent of GFAP+ area that colocalized with TAP1 in the CA1 region of the hippocampus by immunohistochemistry. Indeed, astrocyte TAP1 protein expression was upregulated in EAE compared to normal (Figure 3-3 C-D).

The upregulation of multiple genes involved in MHC I signaling at the RNA level suggested that MHC I expression itself would be upregulated in EAE. Thus, we analyzed the expression of MHC I protein in astrocytes by immunohistochemistry using the pan MHC I marker OX-18[23]. We found increased MHC I expression in astrocytes in EAE compared to normal (Figure 3-3 E-F), consistent with the upregulation of genes upstream of MHC I signaling shown above.

3.4 Discussion

Astrocytes play critical roles in CNS maintenance and response to injury, and play a large part in MS pathogenesis. Here, we took a cell-specific and region-specific approach to understand changes in hippocampal astrocyte gene expression in neurodegenerative disease. We showed that during chronic EAE, the expression of genes involved in interferon signaling and antigen presentation are upregulated in hippocampal astrocytes. Of the genes involved in these pathways, 11/14 are associated specifically with MHC class I signaling; *H2-Q4* and *H2-T23* encode subunits for the MHC I heavy chain, *B2m* encodes the MHC I light chain, and *Tap1*, *Tap2*, *Psmb8* (*Lmp7*) and *Psbm9* (*Lmp2*) are involved in peptide processing for MHC I molecules[24], while Irf9, Ifitm3, Stat1, and *Isg15*, have been associated with increased cells surface MHC I expression[25-28]. In contrast, only *Socs1* has a role exclusively in MHC class II signaling[29], while *CD74* plays a role in both MHC class I and II signaling[30, 31]. *Ifi35* and *Ifit3* are interferon inducible genes, however their direct role in MHC signaling is unknown. The upregulation of these genes corresponded with an increase in MHC I protein expression in astrocytes during EAE. Notably, MHC I expression in astrocytes has been found in MS tissue, inferring that these findings in the EAE model are relevant to the human MS disease[32, 33].

MHC I is canonically known to present antigen to CD8+ cytotoxic T cells. However, during MS and EAE, peripheral immune cell infiltration in the hippocampus is rare, as shown by our lab and others[34-36]. Supporting this idea, MHC I expression in astrocytes at lesion edges and normal

appearing white matter in MS patient tissue was independent of T cell proximity. Together, these studies suggest that MHC I expressed in hippocampal astrocytes during EAE is not functioning to present antigen to CD8+ T cells.

Within the past couple decades, a secondary role of MHC I in the brain has been uncovered. MHC I signaling has been shown to play an essential role in synaptic plasticity during development[37, 38], adult hood[23, 39], aging[40-42], and neuronal recovery[43-45], suggesting a possible non-canonical role of MHC I signaling in astrocytes during disease. While MHC I expression in the CNS is primarily found in neurons and at synapses[23, 46-49], astrocytes can also express MHC I under inflammatory conditions[50, 51]. MHC I in astrocytes can affect synaptic plasticity as shown by Sobue et al who demonstrated an inverse relationship between astrocyte MHC I expression and dendritic spine density in the cortex[50]. Synaptic plasticity can be directly altered by interactions between MHC I and receptors at the synapse such as PirB, CD3ζ-containig receptors, or insulin receptors[47, 52-54]. Astrocyte derived MHC I can signal to these synaptic receptors by direct contact from surface MHC I at astrocyte end feet, or through soluble release of MHC I from the astrocytes[50]. Additionally, astrocyte MHC I can bind to DAP12 on microglia[55, 56] to indirectly affects synapses though the synaptic elimination functions of activated microglia. Identifying the specific receptors and mechanisms relevant to MHC I regulated synaptic loss during EAE may uncover new targets to treat neurodegenerative disease.

Studies examining the role of MHC I in other neurodegenerative models suggest multiple mechanism of MHC I signaling at the synapse depending on the location and type of injury. For example, surface MHC I deficiency (*B2m*$^{-/-}$) increased synaptic stripping and decreased axonal regeneration after sciatic never injury[43], while recovery after stroke was promoted in MHC I deficient mice (KbDb$^{-/-}$)[44]. Notably, these studies were done in global knock out conditions in which MHC I was eliminated during development as well. Thus, it is unclear if the effects of MHC I deficiency were occurring at the time of injury, or if the effects were related to alterations that

occurred during development. Additionally, these two studies differed in the MHC I knock out model used, which may indicate different roles for MHC I co-factors and the MHC I heavy chain in synaptic plasticity. Direct functions of MHC I in the CNS during EAE have not been addressed. While the loss of surface MHC I expression has been shown to exacerbate clinical disease and demyelination, this study used global MHC I knock out mice[57]. Therefore, MHC I was knocked out in the peripheral immune system as well, altering the initial immune response in EAE. This would have confounded CNS specific roles of MHC I during EAE since damage in the CNS is initiated by infiltrating immune cells. A correlation between reduced disease severity and lower CNS MHC I expression has also been shown, however, the direct actions of MHC I in the CNS during disease were not established[58, 59]. In order to determine how astrocyte MHC I expression affects synapses during EAE, we will use the Aldh1l1-Cre/ERT mouse line to conditionally knock out *B2m*, and therefore surface expression of MHC I[60], specifically in astrocytes during adulthood. In the Aldh1l1-Cre/ERT line, *Cre* expression is induced by tamoxifen administration, allowing us to control the timing of *Cre* expression and subsequent gene know out. It is critical that we knock out *B2m* in adulthood so that loss of MHC I during the early stages of neural circuit formation does not affect the neuroinflammatory response in EAE. We will examine how loss of astrocyte MHC I affects microglial activation and synaptic loss in the hippocampus during EAE. These studies will be the first to show an astrocyte specific role of MHC I in synaptic plasticity without confounding developmental effects.

The expression of MHC I signaling genes in astrocytes may be regulated in a sex dependent manner. Indeed, MHC I expression was inversely correlated with estrogen receptor (ER) alpha (ERα) and ERβ expression in normal and cancerous human breast tissue,[61] and estrogen pre-treatment reduced MHC I expression in LPS-induced microglia[62]. MHC I expression was also upregulated in the aging CNS in a sex specific way by which MHC I related genes were upregulated more in females compared to males[63, 64]. Therefore, we were interested to know if

MHC I related genes were also upregulated during EAE in female astrocytes. We found that the genes were upregulated in EAE astrocytes in females, however the degree of gene expression change appeared to be less than in males. Potential sex differences will be further investigated in future experiments using gonadectomy (GDX) prior to sexual maturity to remove circulating adult sex hormones in both males and females. We will use GDX mGFAP-Cre(77.6)xRiboTag mice to determine how sex hormones affect the expression of MHC I signaling genes in astrocytes. A consensus estrogen response element (ERE) is predicted to be located in the *Tap2* gene[65] (Figure S3-1A). *Tap2* is located in the MHC locus of chromosome 17 in close proximity to *Tap1, Psmb8,* and *Psmb9* (Figure S3-1B). Therefore, it is possible that all or some of these four genes may be regulated by the ERE, and loss of sex hormones will affect expression of these genes. Additionally, MHC I genes are largely regulated by the MHC class I regulatory element (CRE) which has been shown to contain a sequence motif similar to EREs[66]. Binding of transcription factors to the MHC CRE has been shown to be mediated by estrogen[67], thus providing another avenue for estrogen interactions with MHC I gene expression. We expect that gonadectomy will alleviate the repressive effects of estrogen on MHC I gene expression, and there will be a large increase in MHC I related genes during EAE in gonadectomized females. GDX males may also have changes in MHC I gene expression since testosterone is aromatized to estrogen in the brain which can act through estrogen receptors[68-70]. However, this effect will likely be less than the removal of estrogens in females.

While additional experiments are needed to investigate a sex difference in MHC I signaling in astrocytes in the hippocampus, our studies do suggest an overall sex difference in the astrocyte response in the hippocampus during EAE. Our previous studies investigating gene expression changes in astrocytes identified very few differentially expressed genes between normal and EAE females in the hippocampus[5]. This is in stark contrast to the hundreds of differentially expressed genes found in males. These two experiments utilized different GFAP-Cre mouse lines, and

therefore further investigation into the sexual dimorphic expression of astrocyte genes in response to hippocampal damage are warranted.

Our gene expression studies in EAE uncovered MHC class I as an important signaling cascade in the neuroinflammatory response of hippocampal astrocytes. The exact role of MHC I during the neuroinflammatory response in EAE needs to be investigated by cell specific knock out approaches after development. Such experiments will verify if excess MHC I signaling in astrocytes exacerbates EAE disease course and synaptic stripping. Future studies investigating sex hormone effects on MHC I signaling will elucidate sex differences in gene regulation in astrocytes. The discovery of MHC class I signaling in hippocampal astrocytes during EAE opens new doors for therapeutic targets in neurodegenerative disease.

3.5 Materials and Methods

Mice. mGFAP-Cre(77.6) (B6.Cg-Tg(Gfap-cre)77.6Mvs/J) and RiboTag (B6N.129-Rpl22tm1.1Psam/J)[22] mice were purchased from Jackson Laboratory and crossed to generate mice that express the HA tag on ribosomal protein RPL22 in astrocytes (mGFAP-Cre(77.6)xRiboTag). Wild type C57BL/6J mice were also purchased from Jackson Laboratory. Animals were maintained under standard conditions in a 12-hour dark/light cycle with free access to food and water ad libitum. All procedures were reviewed and approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

Genotyping. mGFAP-Cre(77.6)xRiboTag mice were genotyped by standard PCR procedures using the following primer sequences: Floxed *Rpl22* (forward: 5'-GGG AGG CTT GCT GGA TAT G-3', reverse: 5'-TTT CCA GAC ACA GGC TAA GTA CAC-3'), *Cre* (forward: 5'-CCG GTT ATT CAA CTT GCA CC-3', reverse 5-CTG CAT TAC CGG TCG ATG CAA C-3').

Active EAE induction. mGFAP-Cre(77.6)xRiboTag mice were induced with EAE as previously described[71]. Briefly, mice were immunized at 8-12 weeks of age by subcutaneous injection of MOG peptide 35-55 (MOG_{35-55}) (200 ug/mouse, Mimotopes) emulsified in Complete Freund's adjuvant (BD Bioscience), supplemented with Mycobacterium tuberculosis H37Ra (200 ug/mouse; Difco Laboratories) over draining auxillary and inguinal lymph nodes on EAE days 0 and 7. Pertussis toxin (500 ng per mouse; List Biological Laboratories, Inc.) was administered on EAE days 0 and 2 by intraperitoneal injection. Animals were monitored for EAE symptoms daily, and scored according to the standard EAE 0-5 scoring system as follows: (0) no symptoms, (1) loss of tail tonicity, (2) loss of righting reflex, (3) partial paralysis of at least one limb, (4) compete paralysis of at least one limb, (5) moribund, as previously described[72].

RNA Coimmunoprecipitation and Isolation. On EAE day 45, EAE mice as well as healthy age- and sex-matched controls were exposed to a lethal dose of isoflurane and transcardially perfused with 1xPBS for 6 minutes followed by 4% paraformaldehyde for 6 minutes. Hippocampal tissues were microdissected, snap frozen, and stored at -80°C until use. Frozen tissues from EAE and matched controls were homogenized as previously described, with modifications[5]. Briefly, hippocampal tissues were homogenized in ice-cold Dounce homogenizers in 1.5 mL of ice-cold homogenization buffer (100 mM KCL, 50mM TrisHCl pH 7.5, 12mM $MgCl_2$, 1% Nonidet P-40, 1mM DTT, 1xproteinase inhibitors, 200 units/mL RNAsin, 100 ug/mL cycloheximide, 1mg/mL heparin). Homogenates were centrifuged at 16,000xg, at 4°C for 20 minutes to remove cellular debris and separate the cytosolic fraction. Anti-HA magnetic beads (HA.11 Clone 16B12, 1:25) were added to the supernatant and incubated at 4°C, rotating, overnight. The solution was placed on a magnet, and the supernatant was removed. The magnetic beads were washed with high salt buffer (300 mM KCl, 50mM TrisHCl pH 7.5, 12mM $MgCl_2$, 1% Nonidet P-40, 1mM DTT, 100 g/mL

cycloheximide) three times, 10 minutes each. The remaining pellet was incubated with Proteinase K (4 mg/mL, Zymo Research) at 55°C for 30 minutes to dissociate the RNA from the ribosomes. RNA was then isolated using the Direct-zol RNA microprep kit following manufacturer's instructions (Zymo Research). Final RNA concentrations were measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific) and RNA quality was assessed by Agilent 2100 Bioanalyzer.

High Throughput RNA Sequencing. RNA sequencing was performed as previously described[73]. Briefly, RNA sequencing libraries were generated by KAPA Stranded RNA-Seq Kit (Kappa Biosystems). Barcoded adaptors were used for multiplexing samples in one lane. Sequencing was performed on Illumina HiSeq3000 for a single end 1x50 run. Data quality check was done on Illumina SAC. De-multiplexing was performed with Illumina Bcl2fastq2 v2.17 program. These procedures were performed at the UCLA Technology Center for Genomics and Bioinformatics.

Quantitative RT-PCR. cDNA was generated using Tetro cDNA synthesis kit (Bioline). Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) in a Bio-Rad 70 Opticon 2 qPCR/Peltier Thermal Cycler at the UCLA-DOE Biochemistry Instrumentation Core Facility. Primer sequences can be found in Table S3-1. Ct values were normalized to *Actb*.

Immunohistochemistry. On EAE day 45, EAE mice as well as healthy age- and sex-matched controls were exposed to a lethal dose of isoflurane and transcardially perfused with 1xPBS for 6 minutes followed by 4% paraformaldehyde for 6 minutes. CNS tissues were dissected and submerged in 4% paraformaldehyde overnight at 4°C. Tissues were then cryoprotected in 30% sucrose for 24 hours, or until the tissues sank to the bottom of the tube. The tissues were then

embedded in 7.5% gelatin/15% sucrose solution and stored at -80°C until use. 40uM thick coronal sections were prepared using a Leica Biosystems cryostat at -25°C and sections were stored in 1xPBS+0.5% sodium azide at 4°C. Sections were washed with 1xPBS two times to remove residual sodium azide before staining. Tissues were permeabilized and blocked with 10% normal goat serum and 0.5% TritonX-100 in 1xPBST (1XPBS + 0.5% Tween-20) for 2 hours. Tissues were then incubated with primary antibody for 2 hours at room temperature followed by overnight incubation at 4°C. The next day, tissues were thoroughly washed with 1xPBST and then incubated with secondary antibody conjugated to cy5, cy3, or Alexa488 (1:1000, Jackson Immuno Research) for 1 hour at room temperature. Tissues sections were then stained with DAPI and washed with 1XPBS before mounting onto slides. Slides were kept in the dark to dry overnight before cover slipping in flouromount G (Southern Biotech) for confocal microscopy. The following primary antibodies were used: mouse anti-HA (1:500, HA.11 Clone 16B12; Biolegend), rabbit anti-HA (1:1000, Invitrogen), rat anti-GFAP (1:500, Life Technologies), rabbit anti-Aldh1l1 (1:1000, Abcam), anti-NeuN (1:500, Abcam), rabbit anti-IBA1 (1:400, Wako), rabbit anti-P2Y12 (1:1000, AnaSpec), anti-CC1 (1:500 , Calbiochem), rabbit anti-TAP1 (1:300, Invitrogen), mouse anti-OX18 (1:400, Biorad), guinea pig anti-PSD95 (1:500, Synaptic Systems), rat anti-MHCII-Cy5.5 (1:400, Biolegend).

Stained sections were imaged using am Olympus BX51 fluorescence microscope with a DP50 digital camera. Images were taken at 40x magnification in 6µm thickness with 1µm stacks. All images were taken using the integrated software program Slidebook4.2 (Intelligent Imaging Innovations) or OLYMPUS cellSens Dimension 2.2 (Olympus Corporation). ImageJ (NIH) was used for image processing and analysis.

Statistical Analysis. All statistical analyses were done in a blinded fashion. Statistical analysis and figure production for RNA-Seq data were performed as previously described above in

Chapter 2[73]. Canonical pathway enrichment analysis was performed for differentially expressed genes in each tissue using Ingenuity Pathway Analysis (QIAGEN, Redwood City, www.qiagen.com/ingenuity). Quantitative RT-PCR and immunohistochemistry data were evaluated by unpaired t-test or non-parametric Mann-Whitney U test (specified in figure legends) using GraphPad Prism 8.2.1, comparing normal and EAE groups in each sex separately. Quantitative RT-PCR data are representative of two replicate experiments on separate sets of normal and EAE mice, and are displayed as means ± SD. Immunohistochemistry data are representative of a single experiment, and are displayed as means ± SEM. Exact p values and group sizes can be found in the figure legends.

Figures

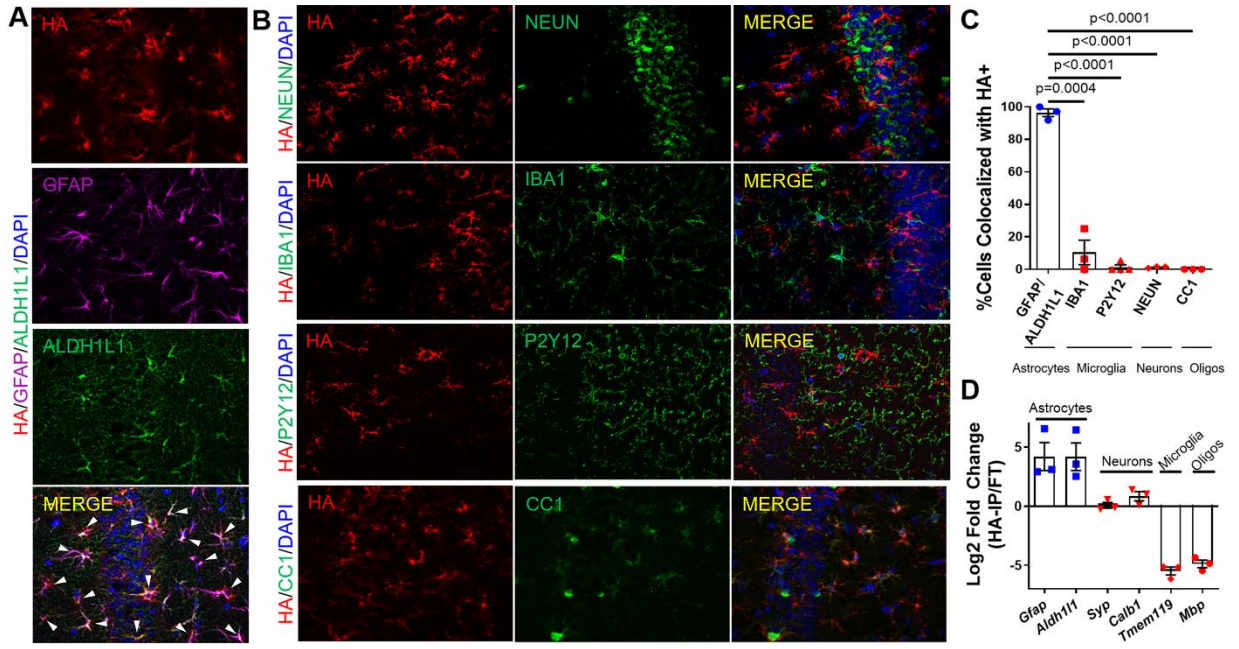

Figure 3-1. Specificity of astrocyte mRNA isolation by HA-mediated immunoprecipitation. Hippocampus CA1 regions from mGFAP-Cre(77.6)xRiboTag mice were imaged at 40x magnification. **(A)** HA expression (red) is colocalized with astrocytes (GFAP purple, ALDH1L1 green). **(B)** HA expression (red) is not colocalized with neurons (NEUN, green), microglia (IBA1, P2Y12, green), or oligodendrocytes (CC1, green). **(C)** Quantification of the number of each cell type that colocalizes with HA expression. N=3, error bars represent SEM. P values were calculated by unpaired t- test. **(D)** Enrichment of astrocyte-specific gene expression (*Gfap, Glial fibrillary astrocytic protein and Aldh1l1*, *Aldehyde dehydrogenase 1 family member l1*), and de-enrichment of neuronal (Calb1; Calbindin 1 and Syp; Synaptophysin) and oligodendroglial (*Mbp*, *Myelin basic protein*) specific gene expression, shown as the $\log_2$ fold change calculated between the RNAs immunoprecipitated by anti-HA antibody versus the flow through.

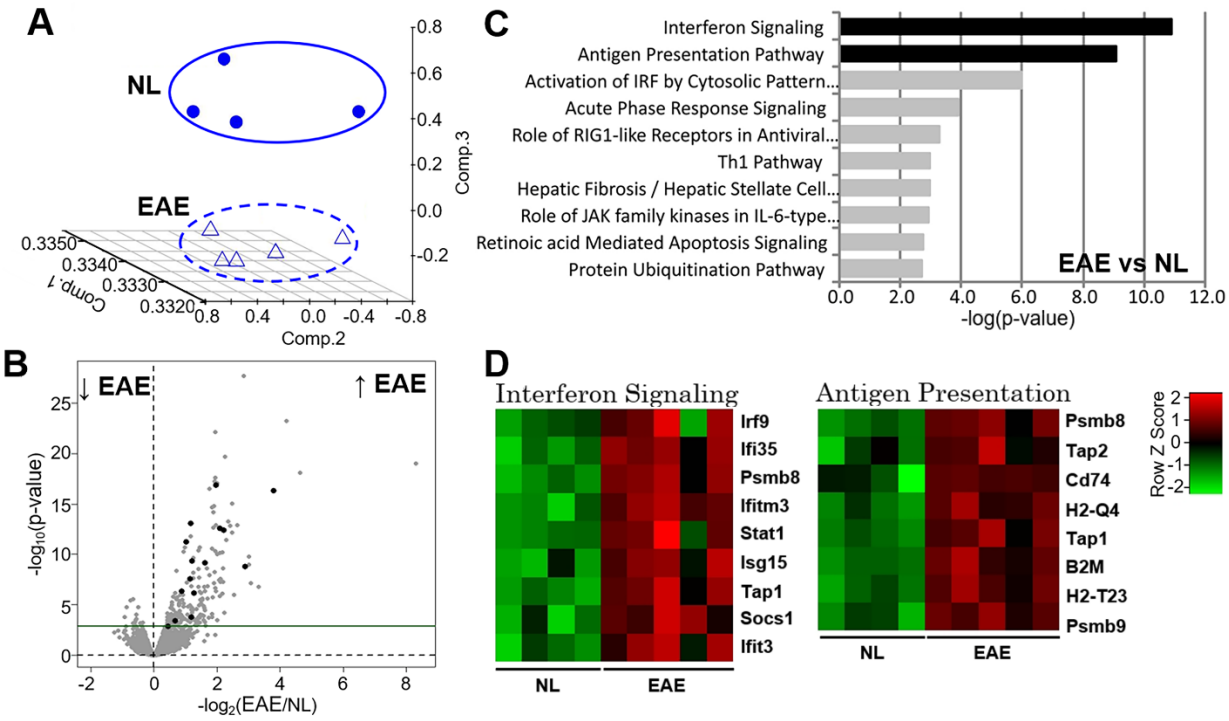

Figure 3-2. Interferon signaling and antigen presentation pathways are upregulated in hippocampal astrocytes during EAE. mGFAP-Cre(77.6)xRibotag mice were induced with active EAE. On EAE day 45, astrocyte specific RNA from the hippocampus was isolated and analyzed by RNA-Seq. **(A)** Principle component analysis (PCA) of normal (NL, n=4) and EAE (n=5) hippocampal astrocytes. **(B)** Volcano plots showed the distribution of differentially expressed genes between NL and EAE hippocampal astrocytes. FDR<0.1 was used as a threshold for significance (green line, R package edgeR). Genes to the right and above the green line have higher expression in EAE compared to normal. Genes to the left and above the green line have higher expression in normal compared to EAE. **(C)** Top 10 differentially regulated canonical pathways from significantly differentially expressed genes between normal and EAE**.** Interferon Signaling and Antigen Presentation Pathway were the top two pathways. **(D)** Heat map shows upregulation (red) of the genes in both interferon signaling and antigen presentation pathways in EAE. These genes are marked with black dots in the volcano plot in **(B)**.

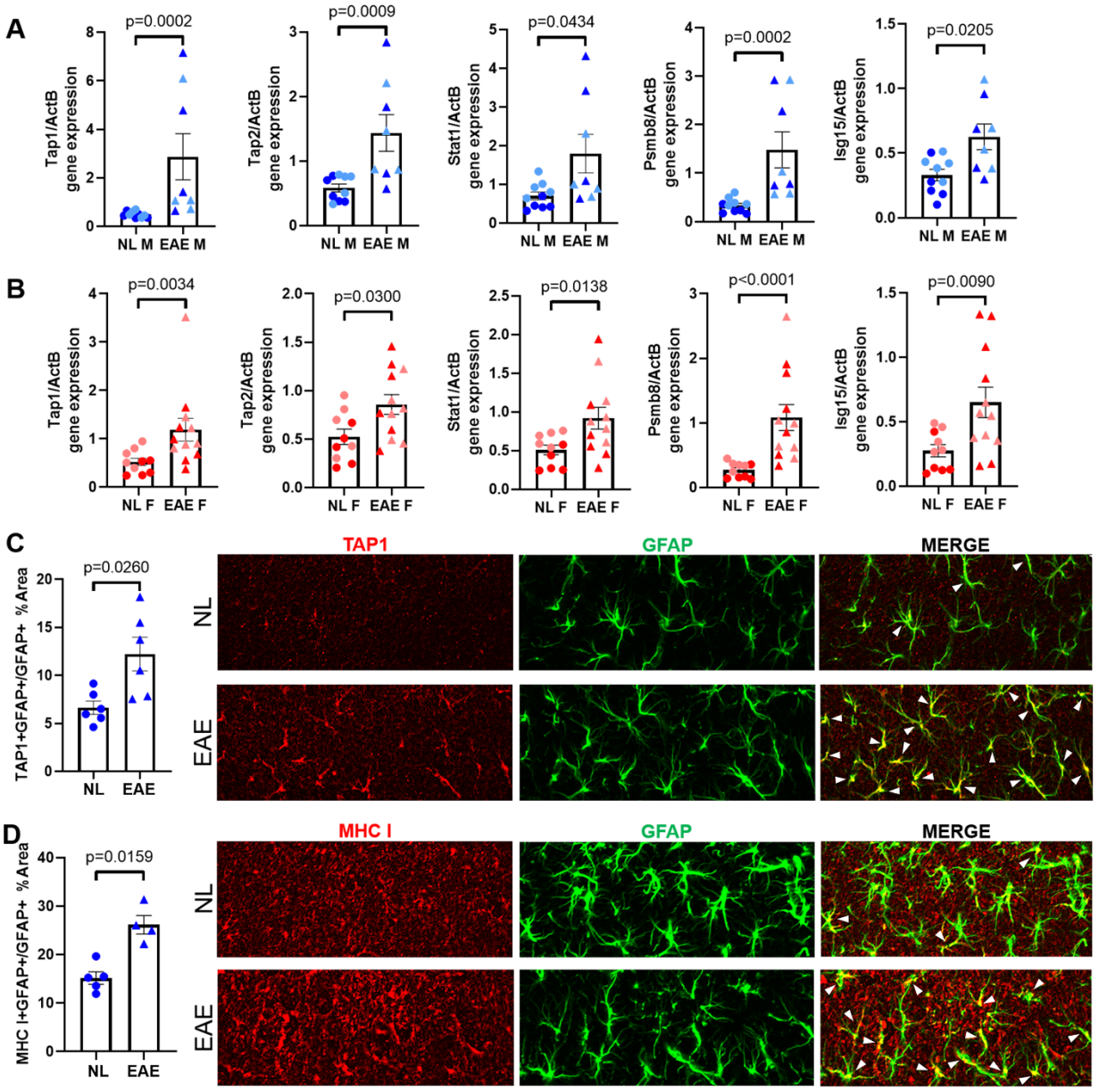

Figure 3-3. MHC class I signaling genes are upregulated in hippocampal astrocytes during EAE. (A-B) Two additional EAE experiments were performed in mGFAP-Cre(77.6)xRiboTag mice. mRNAs were isolated on EAE day 45, and analyzed by quantitative RT-PCR for MHC I related genes (*Tap1, Tap2, Stat1, Psmb8, Isg15*) in normal (NL) and EAE males **(A)** and females **(B)**. Normal male n=10, EAE male n=8, Normal female n=10, EAE female n=12. Data are representative of two separately colored replicate experiments. Error bars represent SD. P-values

were calculated by Mann-Whitney *U* test. **(C-D)** Wild type C57BL/6 mice were induced with EAE. On EAE day 45, brain tissue was collected for immunohistochemical analysis. The percent of GFAP+ area (green) that colocalized with TAP1 (**C**, red) and MHC I (**D**, red) protein expression in the CA1 region of the hippocampus was quantified by immunohistochemistry. Representative images were taken at 40x magnification. EAE mice (n=4-5) had more TAP1 and MHC I protein expression colocalized with GFAP+ astrocytes than NL (n=5-6)**.** Error bars represent SEM. P values were calculated by Mann-Whitney *U* test.

Gene	Forward (5'-3')	Reverse (5'-3')
Gfap	CTG CGT ATA GAC AGG AGG CAG	CTT GGC CAC ATC CAT CTC CA
Aldh1L1	CAC TGG CCG ACT TGA AGA TT	ACC TCC TCA GTT GCA GGG TT
Syp	CAG TTC CGG GTG GTC AAG G	ACT CTC CGT CTT GTT GGC AC
Calb1	CTG TGG GTA AGA CGT GAG CC	GGA GCT ATC ACC GGA AAT GA
Tmem119	CTT CAC CCA GAG CTG GTT CC	GGG AAG AGG CTG AAG AAC CC
Mbp	CTT CAA AGA CAG GCC CTC AG	CCT GTC ACC GCT AAA GAA CG
Tap1	CCA CGA GTG TCT CGG GAA T	ACT GAA GCT GGT AGA GAA CGA A
Tap2	TGC TGT TCT CGG GTT CTG TC	CCC CTT TTT CCC CGA TTT CTG T
Stat1	TCT GGC CTT GGA TTG ACA CC	TGA ATG TGA TGG CCC CTT CC
Psmb8	GAC AAT GGG ACT CGG CTC TC	TGT GGT ACA TGT TGA CGA CTC C
Isg15	AGC AAT GGC CTG GGA CCT AA	AGA CCC AGA CTG GAA AGG GT

Table S3-1. Primer sequences used for quantitative RT-PCR analysis.

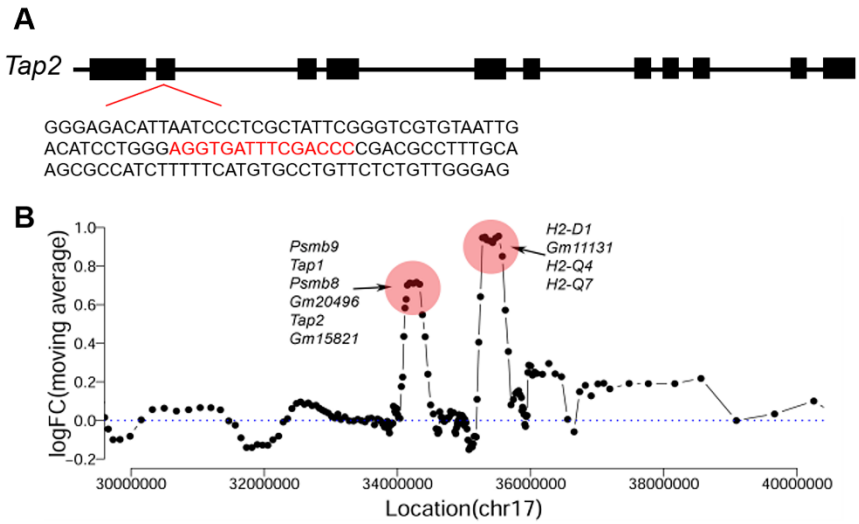

Figure S3-1. Estrogens may regulate MHC I signaling genes though an estrogen response element in *Tap2*. (A) Partial gene sequence of the mouse *Tap2* gene showing the location and sequence of a potential estrogen response element[65]. **(B)** MHC I signaling genes with upregulated expression during EAE are clustered together near *Tap2* on the mouse chromosome 17.

3.6 References

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Chapter 4

Conclusions, Limitations, and Future Directions

Multiple sclerosis (MS) is a complex disease, comprising both autoimmune and neurodegenerative components. In this dissertation, I aimed to further understand mechanisms of neurodegeneration in the immune system and central nervous system (CNS) of EAE as a model for MS. I showed two approaches for investigating molecular mechanisms of neurodegeneration: 1) sex-specific and 2) region-specific and cell-specific gene expression approaches. These two approaches are not mutually exclusive of each other, and should be integrated. In Chapter 2, I primarily took a sex-specific approach to discover parental imprinting as a mechanism by which sex chromosomes alter gene expression. However, these studies were done in a region-specific and cell-specific manner by investigating CD4+ T cells derived from the lymph nodes. In Chapter 3, I primarily took a region-specific and cell-specific approach to show upregulated expression of MHC class I signaling in hippocampal astrocytes. However, since MHC I gene expression is regulated by sex hormones, future experiments will take a sex-specific approach to understand the mechanisms of our finding.

There are known sex differences in MS in which women are more susceptible to MS, while men have a shorter time to disease progression, and have worse cognitive function. Our lab and others have extensively studied the role of sex hormones in the immune response, specifically in EAE, reviewed in Chapter 1. However, the role of sex chromosomes remained largely unknown. Previous studies by our lab using the “four core genotypes” (FCG) model demonstrated that the XX sex chromosome complement is more proinflammatory in immune cells which exacerbates EAE disease compared to XY^{-}. Whether this was due to Y genes, X gene dosage effects, or parental imprinting was not determined. In Chapter 2, I used the FCG to examine gene expression differences in T lymphocytes from XX and XY^{-} mice during the early inflammatory response to further understand why XX is more proinflammatory. While we found a couple X genes with higher expression in XX consistent with X genes escaping X inactivation, we were surprised to find a cluster of five X genes with higher expression in T lymphocytes from XY^{-} compared to XX. This

result was consistently found in autoantigen stimulated T lymphocytes from autoantigen immunized mice, as well as in anti-CD3/CD28 antibody stimulated T lymphocytes from healthy nonimmunized mice, in both females and males. This result suggested a role for parent-of-origin differences in DNA methylation of X genes (parental imprinting), whereby the paternally inherited X chromosome (X_p) had more DNA methylation than the X maternally inherited X chromosome (X_m). Indeed, we found more DNA methylation on the X_p chromosome compared to X_m using a the XY*^{x} mouse model. The XY*^{x} model was critical to these studies since it produces female mice with only one X chromosome, and therefore do not have confounding effects of DNA methylation due to random X inactivation. While the overall effect of the upregulation of these X genes in XY is unknown, this study underlined the importance of studying biological sex variables to understand mechanisms of disease and was the first to show chromosome wide parent-of-origin differences in DNA methylation that affect gene expression.

The availably of the XY*^{x} mouse model was the largest limitation to this study; it was only available on the SJL background. On the other hand, the gene expression studies were done in the C57BL/6. Since these different strains are known to exhibit different forms of EAE disease, it was possible that the role of parental imprinting could differ between the strains. Thus, we also analyzed gene expression of the FCG mice on the SJL background. Again, we found upregulation of X genes in XY compared to XX, suggesting a role for parental imprinting, however the genes were different than those found in C57BL/6. This is likely due to the fact that these strains experience different forms of disease, and thus different genes may be involved. Additionally, we showed that the effects of parental imprinting were not specific to these two strains alone by analyzing gene expression data from F1 reciprocal hybrid crosses in which expression from the X_m and X_p can be determined by single nucleotide polymorphisms between the two strains. In this case, we saw higher X gene expression from the maternal X regardless of which strain it came from. Analyzing parent-of-origin differences in DNA methylation in multiple strains using the XY*^{x}

model would provide a unique outlook on strain differences in gene expression. Similar experiments could also be done on CD8+ T lymphocytes or B lymphocytes to have a comprehensive understanding of how parental imprinting affects all immune cell sub types.

A parallel project investigated how X gene dosage effects could regulate XX susceptibility to proinflammatory responses compared to XY. Using multiple gene expression data sets from T lymphocytes in mice and humans, *Kdm6a*, a known X escapee gene encoding for a histone demethylase, consistently had higher expression in XX compared to XY. Conditional knock out of *Kdm6a* in T cells (*Cd4*-cKO) ameliorated EAE disease and reduced spinal cord pathology. I then characterized changes in T lymphocyte populations in the *Kdm6a Cd4*-cKO and found that T lymphocytes lacking *Kdm6a* had a more naïve phenotype compared to wild type. Notably, this was due to a striking loss of CD44 expression in the *Cd4*-cKO. Analysis of Chip-Seq data revealed that *Cd44* gene expression is directly regulated by KDM6A demethylase activity, indicating that when *Kdm6a* is expressed, repressive histone methylation is removed from the *Cd44* gene allowing expression in memory T cell populations. T lymphocytes lacking *Kdm6a* also had reduced proliferation and proinflammatory cytokine production. Together with our studies of parental imprinting, we show that sex chromosomes play an important role in regulating the immune responses and this can be done either by increased expression of X genes that escape inactivation in females, or parental imprinting effects of genes that do not escape X inactivation. Moreover, all of these factors can be modulated by sex hormones in a complex system that drives the proinflammatory response in females. Our methodologies used here can be applied to other immune reactions and disorders to understand how sex chromosomes alter gene expression in different systems, mouse strains, and even species.

The extensive knowledge of the immune response in MS has resulted in 14 available treatments for MS patients, all of which are immunomodulatory in peripheral blood and lymphoid tissues. These treatments have only mild and indirect effects on neurodegeneration. No

therapeutics exist that directly target neurodegeneration. Therefore, while it is important to understand mechanisms of inflammation in the periphery, it is imperative that we also understand mechanisms of neurodegeneration in the CNS to identify targets for direct CNS intervention. We recently showed the importance of taking a cell-specific and region-specific approach to study gene expression in the CNS, since changes in gene expression during disease not only reflect changes within cells, but also changes in cell composition during disease. In Chapter 3 I focused on understanding gene expression changes specifically in hippocampal astrocytes during the late progressive stage of EAE. I isolated mRNA from hippocampal astrocytes and analyzed it by high throughput RNA sequencing. Our analyses revealed an upregulation of MHC class I related genes during EAE. Notably, these results were confirmed by quantitative RT-PCR in males as well as females, suggesting these genes play an important role in the neuroinflammatory response since it occurs in both sexes. MHC I protein expression was found to be upregulated in astrocytes at the protein level, indicating that MHC I signaling is indeed affected by EAE in astrocytes.

MHC I is required for proper neural circuit formation and synaptic plasticity in development, and it has been shown to play a role in modulating synaptic plasticity in disease as well. Most studies of MHC I in the CNS only report expression in neurons, and investigate mechanism of neuronal derived MHC I at synapses. However, we show here that astrocytes expressed MHC class I and related signaling genes in the hippocampus. While a correlation between MHC I signaling and neurodegeneration has been shown during EAE, direct actions of MHC I on synapses have not been determined. We will conditionally knock out *B2m* in astrocytes to eliminate MHC I surface expression and examine how this affects hippocampal pathology in EAE. This knock out will be done in a time dependent manner using tamoxifen inducible Cre line, Aldh1l1-Cre/ERT, so that synapses and neural circuits during development are not affected. Using tamoxifen for these studies can be confounding since tamoxifen binds estrogen receptors and we know estrogen signaling affects EAE disease course. Therefore, tamoxifen will be

administered two weeks prior to EAE inductions, giving the drug time to be metabolized before disease onset. We will then examine how loss of astrocyte MHC I signaling affects synaptic loss and microglial activation in the hippocampus during the late chronic stage of EAE. Whether loss of MHC I exacerbates or alleviates these pathologies will provide insights to astrocyte mechanism during neuroinflammation.

Since astrocytes interact with neurons, particularly at synapses, it is likely that astrocyte derived MCH I can act through the same receptors and mechanisms as neuronal MHC I. These receptors include, but are not limited to, PirB, CD3ζ, and insulin receptors. Astrocyte MHC I may also signal to receptors on microglia such as DAP12. Antagonism or deletion of the individual receptors during EAE may be sufficient to determine which receptors are most critical for synaptic plasticity regardless of which cell type the MHC I signaling is originating from. However, it is possible that the MHC I signal elicits different responses depending on the cell type origin. Individually regulating each of these receptors with agonists and antagonists in combination with astrocyte specific deletion of MHC I would reveal which receptors are specifically critical for astrocyte MHC I signaling during EAE. To this end, surface MHC I can also be eliminated in neurons and microglia to determine if and how the signaling mechanisms differ between the cell types. This could provide new therapeutic targets to prevent synaptic elimination during neurodegenerative disease.

An interesting and alternative experiment would be to use the *KbDb*$^{-/-}$ knock out in a time and cell specific manner to investigate mechanisms of total MHC I loss during EAE. Since the *B2m* knock out only causes surface MHC I deficiency, complete loss of MHC I expression may have additional effects.

In addition to our conditional knock out studies, future experiments also include investigating how sex hormones affect the astrocyte response during EAE. We found that MHC I related gene expression was upregulated during EAE in males and females, however, this does

not eliminate the possibility that the expression of these genes is regulated by sex dependent factors. In fact, MHC I expression has been shown to be inversely correlated with estrogen expression, and a potential estrogen response element has been found in the MHC region of chromosome 17 where several MHC I genes are located. We aim to 1. determine how the genome-wide astrocyte translatome is altered by loss of sex hormones, and 2. determine how expression of MHC I related genes is altered by sex hormones. I will use gonadectomized male and female mice. These mice will have their sex organs (female, ovaries; male, testes) removed between four to six weeks old, prior to sexual maturity. Thus, these animals will not have circulating sex hormones during adulthood. It is expected that gonadectomy of either sex will increase MHC I expression since testosterone is converted to estrogen in the brain. However, this effect will likely be larger in females. These studies will determine if there is a sex difference in the regulation of MHC class I gene expression. These results may provide important mechanistic information for therapeutic treatments of neurodegenerative disease.

While we took a region-specific approach to gene expression studies by analyzing the hippocampus, there is likely still high levels of heterogeneity among astrocytes across the hippocampus. For example, the dorsal (posterior in humans) hippocampus functions mainly in learning and memory, while the ventral (anterior in humans) hippocampus is involved in mediating emotional responses. Therefore, a more accurate examination of neurodegenerative responses related to hippocampal dependent memory loss should examine only the dorsal hippocampus. While our RNA expression studies utilized the entire hippocampus, the immunohistochemistry assessments of protein expression were focused on dorsal hippocampal sections. Thus, our findings in whole hippocampus were recapitulated in the dorsal segment. This does not rule out the possibility that other important pathways specific to the dorsal segment were overpowered by pathways shared among all hippocampal regions. Additionally, within the dorsal region of the hippocampus there may be heterogeneity in astrocyte responses between sub regions such as

the CA1, CA3 and dentate gyrus (DG). While current immunohistochemical analyses were performed in the CA1 region, additional and future analyses could compare all three regions to identify regional differences within the dorsal hippocampus.

Single-cell RNA-Seq poses and intriguing alternative to our RiboTag gene expression studies. Using single-cell RNA-Seq in combination with our region-specific approach would shed light onto astrocyte heterogeneity within CNS regions. This may lead to the identification of astrocyte subpopulations that could further enhance our ability to identify important pathways regulated during neurodegenerative disease.

The studies presented in this thesis used the animal model for MS as a selected neurodegenerative disease with a strong neuroinflammatory component. However, the results presented here are not limited to MS and EAE. In fact, our findings of parental imprinting and X gene dosage effects in T lymphocytes are able to be extrapolated to the general immune response, as well as other autoimmune diseases, since our findings were also replicated in healthy stimulated cells. Additionally, our findings of MHC I signaling in astrocytes likely contribute to neuropathology of other neurodegenerative diseases such as stroke, ALS, Alzheimer's and normal aging, since MHC I is known to be upregulated in all of these conditions.