THE IMPACT OF EARLY LIFE ADVERSITY ON MAST CELLS

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ABSTRACT

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Exposure to early life adversity (ELA) is a significant risk factor in later life susceptibility to inflammatory disorders, including allergy, asthma and chronic pain disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Mast cells (MCs) are innate immune cells known to play central roles in inflammatory disorders and have been linked in the pathophysiology of stress-induced diseases. The mechanistic link between ELA and later life MC disease is poorly defined. The aim of this study was to investigate whether ELA impacts long-term MC activity and MC mediator content and additionally, determine whether ELA increased adult MC disease susceptibility. The objective of this dissertation was to determine the long-term impact of early life adversity on mast cell phenotype and function. To answer these questions we utilized a validated early life adversity model of maternal separation and early weaning in mice to further subject to various mast cell disease models. Additionally, we collected bone marrow derived mast cells from these animals to further analyze their phenotype.

The results presented here demonstrate that ELA induces long- lasting changes in MC hyperactivity, mediator content and responses in addition to vulnerability to later life stress and clinical GI dysfunction, with females more at increased risk. Future research will be aimed at elucidating the mechanisms through which ELA programs MC development and function and its role in exacerbating later life GI and allergic disease models.

This dissertation is dedicated to God almighty and all my family.

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First, I want to thank Dr. Adam Moeser; thank you for allowing me to be part of your lab. Thank you for your generous support and mentorship. It has been indeed an honor. Being in your lab has shaped me profoundly; I've gained enormous knowledge not only in the academic aspect, like data presentation, data analysis, critical thinking, and literature review. But also personally, thank you for always pushing me to be a better person and scientist. Thank you for teaching me how to overcome my fears and get out of my comfort zone; thank you for teaching me not to settle and always find ways to improve.

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KEY TO ABBREVIATIONS

ACTH- Adrenocorticotropic hormone

BME –Mercaptoethanol

BM- Bone Marrow

BMMC-Bone Marrow-Derived Mast Cell

BSA –Bovine serum albumin

Ca – Calcium

CCL1-CC Chemokine

CCL2 – Chemoattractant protein-1

ChAT – choline acetyltransferase

CNS- Central Nervous System

CRF- Corticotropin Releasing Factor

DNP- Dinitrophenyl

ELA- Early Life Adversity

ENS – enteric nervous system

EW – Early Weaning

FD4 – fitc4kDa dextran

FGID- Functional Gastrointestinal Disorder

FYN- Proto-oncogene tyrosine-protein kinase

GI- Gastrointestinal

GPR- G protein-coupled Receptor

HPA- Hypothalamic–Pituitary–Adrenal

IBD- Irritable Bowel Disease

IFN-γ – Interferon gamma

IL1 – Interleukin 1

IL10 – Interleukin-10

IL3- Interleukin 3

IL33- Interleukin 33

IL6 – Interleukin 6

ISO – Isoproterenol

LPS- Lipopolysaccharide

LYN- Src Family Tyrosine Kinase

MAPK- mitogen-activated protein kinase

MCP-1 – Monocyte chemoattractant protein-1

MCs- Mast Cells

MMP- metalloproteinases

MRGPRX2- Mas-Related G Protein-Coupled Receptor

MS- Maternal Separation

NFKB- Nuclear Factor kappa-light-chain

NH- Normal Handled

NK- Natural Killer Cells

NMS- Neonatal Maternal Separation

NMSEW- Neonatal Maternal Separation plus Early Weaning

PAMP- Pathogen-associated molecular patterns

PD-Postnatal Day

pMC- Peritoneal Mast Cell

PPAR- peroxisome proliferator-activated receptors

PPARα – Peroxisome proliferator-activated receptor alpha

PPARγ – Peroxisome proliferator-activated receptor-γ

PVN- paraventricular nucleus

RS – restraint stress

S1P- Sphingosine-1-phosphate

SCF-Stem Cell Factor

SHRP- Stress Hyporesponsive Period

ST2- IL-1 receptor family

STIM1- Stromal interaction molecule 1

TGFB- Transforming growth factor beta

TLR- Toll-like Receptor

TLR2- Toll-like Receptor 2

TLR3- Toll-like Receptor 3

TLR4- Toll-like Receptor 4

TLR7- Toll-like Receptor 7

TNFα- Tumor Necrosis Factor alpha

VCAM- vascular cell adhesion molecule

VEGF- Vascular Endothelial Growth Factor

WAS-Water Avoidance Stress

WHO- Word Health Organization

CHAPTER ONE

Literature Review

Impact of Early Life Adversity on Immune Development and Immune Responses

Prevalence and Relevance of Early Life Adversity

Exposure to early life adversity (ELA), such as, abuse, neglect, violence, parental loss, has shown to be associated with increased susceptibility to many diseases later in life including chronic inflammatory disease, cardiovascular disease, migraines headaches, diabetes, obesity, cancer, and overall premature mortality [5-10] . Particularly, it is of great concern as the number of child mistreatment and abuse is steadily increasing. In the United States alone, 700,000 cases of abuse are reported annually; in the U.S, 1 in 4 children experience some abuse or neglect [11]. According to the National Center of Child Abuse and Neglect, close to 7.8 million child abuse cases are reported annually, declaring a public health problem with long-term economic consequences of $428 billion annually [11-13]. Furthermore, childhood adversity encompasses all creeds, socioeconomic levels, and cultural groups [11, 14]. The impact of childhood adversities is additive in that as the number of adversities increase, so does the risk for health problems. The World Health Organization and World Mental Health revealed that more than 50% of the total adult population had been exposed to at least one or more childhood adverse events [14]. Despite the high prevalence of ELA and the established negative impact on disease risk across the lifespan, there is still a critical gap regarding the cellular mechanisms by which ELA is increasing disease risk in later life.

Impact of Early Life Adversity on the Immune System

The immune system might play an integrated role in the etiology of these diversified diseases associated with ELA. The immune system is divided into innate and adaptive system. The innate system has found to be involved in the first line of defense against pathogens and other environmental insults; it is unspecific, it has no memory and is evolutionary conserved. It comprises barriers such as the skin and mucous membranes and immune cells such as basophils, mast cells, dendritic cells, macrophages, neutrophils and NK cells among others. The adaptive immune system, on the other hand, is very specific, critical for long-term memory against pathogens; it is composed of specialized cells including B and T cells. [15-17]. Both systems act synergistically when it comes to fighting an infection, the innate response is critical to initiate an adaptive response, while the adaptive helps the innate system clear pathogens in a more efficient and direct manner. Accumulating evidence in literature shows the profound long-term effects of ELA in the stress response system and activation of the immune system [18, 19]. For instance, women with a history of early life adversity show increased ACTH levels and cortisol levels due to CRF hypersecretion leading to long-term disease susceptibility [20]. Similarly, Carrion et al., 2002 demonstrated heightened, adrenal activity and increased cortisol levels in girls with PTSD [21]. High cortisol levels were reported in children reared in Romanian orphanages living in deplorable conditions [22]. Police recruits with a history of ELA had increased catecholamine responses after being subjected to physiological stress [23]. Moreover adverse experiences in childhood have been associated with increased white blood cell counts [24].

Early life adversity shapes overall health and immune phenotype in the early years, when the foundations for early allergic sensitizations take place, usually between birth and early years of age [25, 26], however ELA does not appear to affect all aspects of the immune system, and more evidence shows that ELA affects specific aspects of both innate and adaptive systems, yet the underlying molecular and cellular mechanisms remain unknown. Perhaps one of the most robust discoveries on the long-term effects of ELA on the immune system is the increased levels of circulating pro-inflammatory cytokines and inflammation markers that have been found consistently in different cohort studies

For instance, Baumeister and colleagues found that people exposed to childhood trauma had increased IL-6, TNFα, and C-reactive protein (CRP) in adulthood [4]. Similarly, Miller and Packard reported increased pro-inflammatory markers (IL6 and CRP) in adults previously exposed to ELA [7, 27]. Similarly, Danese et al., 2007 found in a longitudinal- prospective study twice the amount of CRP levels in abused children, compared to non-abused which was further associated with a predisposition for developing cardiovascular diseases in their early adulthood [28-32]. Furthermore, ELA has been shown to induce inflammation in the brain and periphery, [33, 34]. Kiecolt et al., 2011 demonstrated that ELA increased the amount of inflammatory markers IL6 and TNFα, and accelerated cellular aging (shorter telomere length) translating to a 7-15 year lifespan difference in older adults with a history of early life adversity and increased susceptibility to an adult chronic stress, and dementia in the caregiving family.

Although a robust immune response and inflammation is necessary to maintain healthy conditions and to fight off pathogens, persistent chronic low-grade inflammation

can be detrimental to the host and deteriorate health rapidly, leading to a higher susceptibility to disease and this is thought to be the main underlying pathophysiologic factor driving increased disease risk. For instance, high levels of inflammatory markers such as CRP, TNFα, and IL6 have been highly associated with the development of cardiovascular diseases, type 2 diabetes, allergies, periodontal disease and are predictors of poor quality of life, morbidity, and mortality in older adults [35]. Increased immune cell responsiveness or activation may be contributing to the increased inflammatory status in individuals who had been exposed to ELA, as many of the circulating inflammatory cytokines and markers are produced by cells of the immune system. For instance, Miller and Chen, 2009, 2010 found increased expression of NF-KB, and increased levels of the proinflammatory cytokine IL-6 in a bacterial challenge performed to white blood cells of children raised in harsh conditions [27, 36]. In addition to immune cells being affected by ELA and being more reactive, they could also be in a state of low-grade activation or stimulation. Supporting this, Boeck et al, 2016 found increased spontaneous IL-6 cytokine secretion in cultured PBMCs [37] of women with different levels of maltreatment experiences in form of abuse and neglect during their childhood [38]. Together these studies demonstrate that ELA produces long-lasting changes in the immune response systems leading to increased susceptibility to inflammatory diseases in adulthood.

Table 1. Human cohort studies indicating the influence of early life adversity on immunological markers

Reference	Immunological measurements	Relevant findings	Participant information and sample size
(Ehrlich et al 2016)	Production of IL-6 in monocytes stimulated with LPS	ELA associated with increased inflammatory phenotype. ↑IL-6 ↓ Glucocorticoid sensitivity.	Adolescent girls with history of ELA (n = 147)
(Miller and Chen, 2010)	Production of IL-6 in PBMCs stimulated with LPS	↑IL-6 over time ↓ progressive glucocorticoid sensitivity	Females 5 to 19 years old with Harsh family environment (n = 135)
(Baumeister et al, 2016)	CRP, IL-6 and/or TNF-α	↑ Baseline CRP levels Specific inflammatory profiles depending on the type of trauma (sexual, physical or emotional) Significant association between childhood trauma and CRP, IL-6 and/or TNF-α.	Meta-analysis of human participants with any trauma experienced before age 18 females and males 16 870 participants for CRP, 3751 for IL-6 and 881 for TNF-α
(Danese et al, 2006)	WBC, CRP, fibrinogen,	↑ CRP levels 20 years later, in adulthood. ↑Fibrinogen ↑ white blood cell count Children in the definite maltreatment group were 1.86 times more likely to have elevated CRP.	Life-course association between childhood maltreatment and adult inflammation in a birth cohort followed to age 32 years Males (n= 1,037)

Table 1 (cont'd)

Reference	Immunological measurements	Relevant findings	Participant information and sample size
(Miller and Chen, 2009)	Production of IL-6 in PBMCs stimulated with LPS Immune related genes measured in PBMCs by qRT-PCR	In the low early-life SES ↑Genes coding for inflammatory mediators IL1A, CCL2, CXCL2, and CCL20 ↑Genes OLR1 and GPR132, which facilitate macrophage uptake of oxidized low-density. lipoproteins, and of ADM, which regulates vascular tone. ↑Expression of transcripts bearing response elements for NF-KB, and ↑ IL-6. Differences between TLR4 vs TLR5 activation. ↑ IL-6 response to flagellin in adults raised in low SES produced and 51% more in response to Poly I:C	Healthy adults with low or poor early socioeconomic status SES (n = 53) and high early SES (n = 50) (ages 25–40)

Table 1 (cont'd)

Reference	Immunological measurements	Relevant findings	Participant information and sample size
(Kiecolt-Glaser et al, 2011)	IL-6, TNF-α, and telomere length, a measurement of cell aging	↑ IL-6 and TNF-α levels relationship increased in caregivers compared with controls. Multiple childhood adversities was related to both ↑ IL-6 and shorter telomeres. Abuse + caregiving status were associated significantly and independently associated with ↑ depressive symptoms.	58 caregivers for a spouse or parent with Alzheimer's disease or another progressive dementia (mean age = 70.10 years) 74 similar controls (mean age = 69.37 years) who had no caregiving responsibilities Predominantly females Participants completed questionnaires about childhood abuse and other adversities, depression, health, and health behaviors.
(Bartlett et al 1996)	Leukocyte counts and levels of both phagocytosis and killing of *Staphylococcus aureus* by PMNs.	↓ Bacterial killing of Staphylococcus aureus in children with separated or divorced parents, independent of depression. Females associated with ↓ numbers of bacteria phagocytized.	Children who experienced parental divorce and separation and where depressed. (n= 11) Controls (n= 11)

Table 1 (cont'd)

Reference	Immunological measurements	Relevant findings	Participant information and sample size
(Birmaher et al, 1994)	WBC count NK cell activity Lymphocyte proliferation	↓ NK cell activity. Correlated with higher number of adverse events. No difference in WBC count or lymphocyte proliferation	Depressed adolescents with history of ELA (ages 11- 18) and controls (n=17)
(Lemieux et al, 2008)	T cell activation by immunofluorescence CD4+CD45RA+	↑ Percent of positive T cells expressing CD45RA in previously abused woman and with later PTSD.	PTSD woman sexually abused in early childhood and controls (n=12-24), ages 18-40
(Elwenspoek et al, 2017)	Blood stimulated PHA-M (PHA), PWM, or LPS Relative activation status of NK, B cells, and T cells	Overall ↑ immune activation in individuals with ELA, in which T cells are affected. ↓ CD25+ CD8+ T cells ↑ HLA-DR+ CD4 and HLA-DR+ CD8 T cells ↑ CCR4+ CXCR32CCR6+ CD4 T cells	(n =)115 young adults, (n=42) with a history of ELA and (n= 73) who were raised by their biological parent (controls)

Animal Models of ELA and Immune System Effects

Several paradigms have been proposed and studied to assess the effects of postnatal stress in the adult offspring: Maternal separation, maternal deprivation or nutritional deprivation, early weaning, neonatal cold stress, hormonal treatments, bacterial antigens, inflammatory cytokines, neuropeptides. For this section, we will focus on the effects of long neonatal maternal separation on adult offspring's immune function. During the postnatal period, the brain and gut are still developing. Maternal presence is crucial in maintaining the HPA axis normal activity. During the first weeks of life, the pups show a diminished HPA activity in response to stressors, a stress hyporesponsive period (SHRP) [39-42]. Disruption of the maternal-pup bond during this period has long-lasting endocrine consequences, including an increased HPA hyperactivity with an impaired glucocorticoid negative-feedback control of the HPA axis. Early interactions with the mother are crucial for immunological, behavioral, and physiological development. Persinger et al., 1992 demonstrated that early handling and maternal separation in rats produces permanent effects on the immune system and that this persists into adulthood. Pups early handled and maternally separated from their mother from PD 5-20, and later injected at ~10 weeks of age with human serum albumin (HSA) showed an increased antigen-binding capacity of immune cells, which is the number of monoclonal antibodies it will bind which correlates with the number of antigens expressed on immune cells. [43]. This study demonstrates that a disruption in the mother-infant bond increases antigen-binding capacity therefore increasing the possibility of developing allergies in adulthood when exposed to a novel antigen. Most of the literature studying ELA in animal studies has focused on the effects of prenatal

stress and fetal outcomes. A well-known animal model to mimic early life stress is the neonatal and maternal separation model (NMS) in rodents [44-46]. The NMS model is based on the pioneering work of Levine, Denenberg, Meany, and Plotsky, in which stress during early postnatal periods produces long-lasting consequences in stress responsiveness. The most common neonatal maternal separation model consists of daily 3-hour separation ~2- 16 days. Short periods of separation mimic natural rearing conditions; thus, a brief 15 min maternal separation has been found to be protective. Extended periods of maternal separation has shown to increase stress-induced corticosterone levels and hypothalamic levels of CRF; thus, longer periods of maternal separation are required to study the impact of ELA on long-term health outcomes [47]. In this review, we will focus on the longer NMS-model. Long NMS consists of 3 h maternal separation between PD2-14 or PD4-20 and mouse pups between PD 1-14 or 1-18 days of age. Neonatal maternal separation (3h/day at 4-21 days) predisposes adult rats to GI dysfunction in response to mild acute stress. Modification of the protocol by including separation periods plus early weaning increases anxiety and behavioral despair in mice. Further, it has been shown to lessen the buffering effect of maternal care [48].

Early life adverse events, especially disruptions in maternal care and maternal contact, in this early stage of life, produce profound, long-lasting consequences in the offspring. Over the last decades, several early life stress models have been developed and established to study animal behavior and neurological outcomes. Models of early separation include maternal Separation (MS) and early deprivation model. Both models involve the separation of pups from their mothers for either a short period (15min- 1 h)

or long periods (3h - 24 h) [42, 49, 50]. In the early deprivation models, pups are socially isolated, separated from their littermates and mother; in the MS model, pups remain together as a litter. In most studies, pups are separated during the stress hyporesponsive period between PD 4-18. However, the long-term impact of NMS on immune cells of adult animals is very limited. During the postnatal period, basal corticosterone levels, hypothalamic CRH, and pituitary ACTH levels are generally low [42, 51]. Further subjection of pups to mild stressors elicits a blunted pituitary-adrenal response, whereas, in adults, mild stressors will usually provoke high corticosterone levels [51, 52]. Therefore, it has been proposed that the brain is being protected from high levels of glucocorticoids during this stress hyporesponsive period [40]. During the first three weeks of life, pups are entirely dependent on their mothers for warmth, food, physiological homeostasis, and waste removal; thus, separating them from their mother is extremely stressful, and separation during this period can produce long-term dysregulations in the HPA axis responsiveness. In response, pups show elevated corticosterone basal levels and high corticosterone in response to additional stressors such as food deprivation, exposure to novelty, and restraint stress [39, 46, 53-57]. Extensive studies support and validate the MS and early deprivation as models of early life stress. While the effects of NMS on HPA stress responsiveness has been well-studied, comparatively less research has been done to study ELA-associated inflammation.

Neonatal Maternal Separation (NMS) and the Immune System

Animals subjected to NMS have increased colorectal sensitivity and immunological abnormalities, including increased mast cells (MCs) and increased MC relevant cytokines such as NGF, IL4, IL6, and IFNγ IL1-B [8, 58-60]. Meagher et al., 2010 utilized a mouse model of multiple sclerosis; in his study, Balb/cJ mouse pups separated from their dam were later infected in adolescence with Theiler's virus to induce encephalomyelitis and the response to infection was measured in adulthood. Their results showed that NMS increased viral load and hindered viral clearance in the spinal cords of mice, and blunted their corticosterone responses. In contrast, short-term maternal separation of 15-min increased the rate of viral clearance in females [45]. These results demonstrate that NMS increases susceptibility to CNS infection in adulthood and suggest that reduction in HPA sensitivity impacts immune responses to infection. Also, it reflects the contrast in responses between brief and prolonged maternal separation supporting the protective effects of brief periods of maternal separation or short periods of handling seen by previous studies [61, 62]. Avitsur et al., 2006, demonstrated that in male and females adult mice NMS increased pro-inflammatory cytokines IL6, IL1, TNFa, and increased IL-12 and IFN-ɣ in the lung in response to an influenza virus challenge [63]. Their experiments, Sternberg et al., 1989 utilized LEW/N Female rats that develop arthritis in response to group A streptococcus cell wall and in contrast F344/N do not develop arthritis to the same stimulus. They evaluated the HPA axis function and inflammatory responses by measuring early ACTH and corticosterone response to stimuli to determine the link, they used glucocorticoid antagonist RU486, showing that HPA axis was defective in LEW/N. They concluded that

susceptibility to inflammatory diseases, including arthritis, is tightly linked to a dysfunctional HPA axis and production of corticosterone in response to inflammatory and other stress induced mediators [64]. Therefore, a functional HPA-axis-immune system feedback loop is necessary to confer resistance to disease. [64]. Furthermore, long-lasting alteration in early socially deprived macaques showed decreased survival rate, with males being more vulnerable to death. Early social deprivation-induced an increased NK cell number and NK activity and changed lymphocyte patterns compared to non-isolated. These results indicate the impact of early social deprivation in mortality rate and adult immunological status [65]. In contrast, a reduction in lymphocyte proliferative responses and NK activity was observed in maternally deprived monkeys [66]. This shows the maladaptive immune responses induced by early life stress and demonstrates that influence of early life stress on immune responses is likely multi-factorial and dependent upon species, the immunological markers measured, the timing of immune development, and the timing of application of the stressor [67]. Neonatal daily mild stress induced long-term immune changes in adulthood. Loizzo and Colleagues found that repeated exposure to stress enhanced the proliferative capacity of splenocytes with increased Th-1 associated cytokines and increased NK activity. Changes induced by early life stress were prevented when injected with opioid antagonist naloxone. These results demonstrate the relationship between the endogenous opioid systems and stress hormones and that initial pharmacological treatment can ameliorate the long-term immunological effects of early life stress [68, 69] Error! Reference source not found.These studies demonstrate that the long NMS model nduces low-grade chronic inflammation with increased immune reactivity, altered stress

response, and increased susceptibility to inflammatory diseases later in life. Thus it is a valid model to assess ELA-induced long-term disease susceptibility and associated cellular mechanisms.

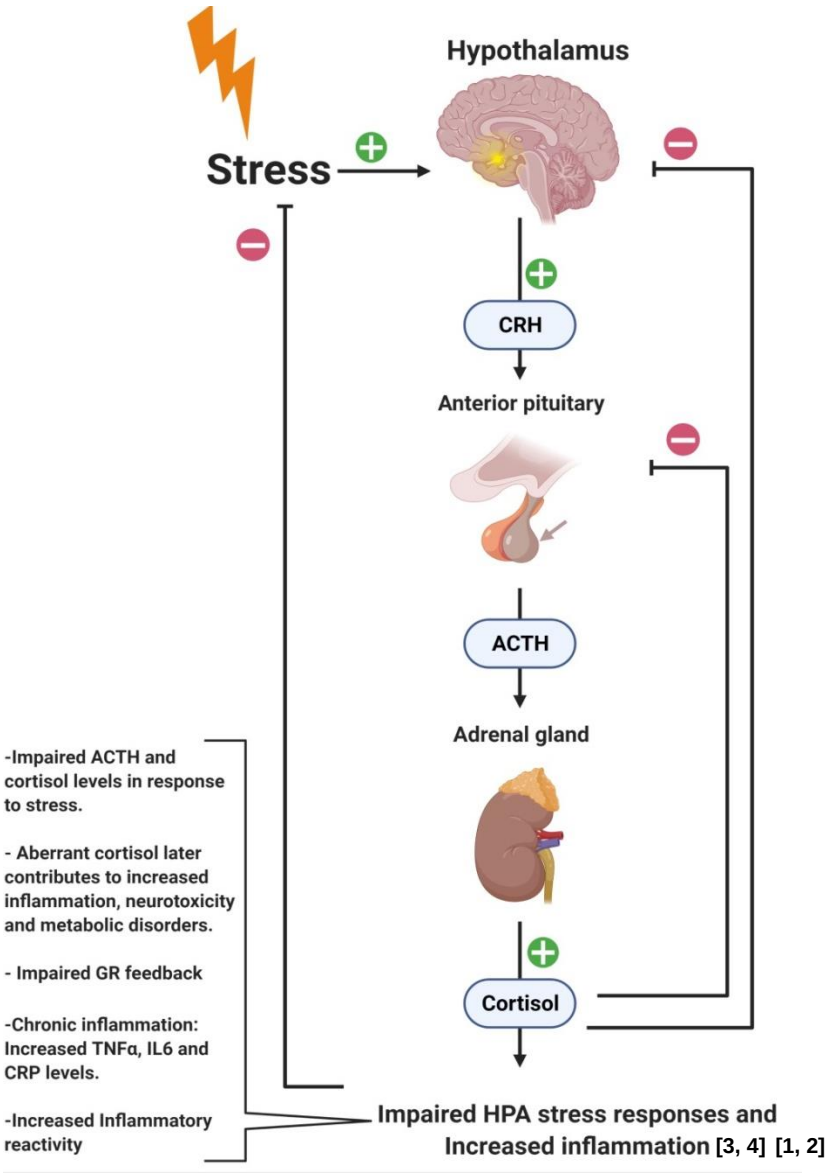

Figure 1.1 Hypothalamic- Pituitary- Adrenal Axis. *Neurological and endocrine response to stress and the Neurological and Immunological changes induced by Early Life Stress.*

Stress in the form of early life adversity induces long lasting behavioral effects, inducing changes in the HPA-axis and affecting the immune system.

Table 2. Animal studies and models of early life adversity indicating the influence of neonatal maternal separation as a model of ELA on immunological cell status

Reference	Type of ELA Model	Relevant findings	Immunological measurements	Animal and sample size
(Persinger et al, 1992)	2-hit model *Postnatal days 5-20* Early handling (genitals stroked for 2 min) + Maternal separation (20 min) + *Postnatal day 80* Huma serum albumin (HAS) challenge	↑ Antibody capacity, ELA affects humoral immunity that persists in adulthood.	Leukocyte antibody capacity measured after challenge at 100 days of age.	n= 36 male, n= 33 female Wistar albino rats
(Meagher et al, 2010)	2-hit model *Postnatal days 2-14* 180-min maternal separation and 15-min maternal separation. *Postnatal day 28* intracranial inoculation with strain of Theiler's virus: a model for multiple sclerosis	180-min MS ↑ viral load and ↓ viral clearance in spinal cords 15-min MS ↑ viral clearance in females in spinal cords ↓ Corticosterone responses on both 15 and 180-min MS ↓Body weight both 15 and 180-min	CNS viral titers in brains, spinal cords, thymus, spleens, and adrenals Corticosterone	Females and males control n= 38, 15-min MS n= 60, 180-min MS n= 38 Balb/cJ rodents

Table 2 (cont'd)

Reference	Type of ELA Model	Relevant findings	Immunological measurements	Animal and sample size
(Avitsur et al, 2006)	2-hit model Maternal separation 6h/day *Postnatal days 1-14* + Adult influence viral infection, at 8-10 weeks mice infected intranasally with HAU influenzza A/PR8 virus	MS induced ↑ lung pro-inflammatory cytokine expression, IL-6, IL-1β, TNF-α. MS ↑ lung IL-1α gene expression in influenza infected mice ↑IL-12 and IFN-γ No effect ion IL-18 Heightened cytokine responses observed in maternally separated females	Lung cytokine gene expression and protein content	Females and males C57BL/6 rodents
(Lewis et al, 2000)	Early social deprivation and maternal deprivation for the first 9 months of life	Early social deprivation ↑NK cell number and NK ↑activity. ↓Survival rates in socially deprived macaques. Changes in lymphocyte patterns ↓ Ratio of helper to suppressor/ cytotoxic T cells (CD4/CD8)	Peripheral blood T, B, and natural killer lymphocytes, macrophages, and monocytes were measured by flow cytometry. Functional cellular immune activity and lymphoproliferative responses	Rhesus monkeys (*Macaca mulatta*) (n=48)

Table 2 (cont'd)

Reference	Type of ELA Model	Relevant findings	Immunological measurements	Animal and sample size
(Laudenslager et al, 1982)	14 day maternal separation	Early separation 🡻 Lymphocyte proliferation in response to mitogens concanavalin A (ConA), phytohemagglutinin (PHA). Levels returned to normality once reunited	Lymphocyte proliferation	Bonnet monkeys (*Macaca radiate*) (n=2)
(Loizzo et al, 2002)	2-hit model *Postnatal days 1-21* Maternal separation + Mild stressor (daily injection with distilled H_2O)	MS and neonatal mild stressor induced selective and long term modulation of immune responses. *Stimulated splenocytes:* 🡹Th-1 cytokines 🡹IL-2, 🡹IFN-γ 🡹TNF-α 🡹Basal NK activity and after stimulation 🡹 Basal splenocytes proliferation and after stimulation 🡻Th-2 cytokines 🡻IL-4, 🡻 IL-10	At day 110 NK activity assessment Measurement of pro-inflammatory cytokines	Male CD-1 rodents

Table 2 (cont'd)

Reference	Type of ELA Model	Relevant findings	Immunological measurements	Animal and sample size
(Fernandes et al, 2021)	2-hit model 3 hours maternal separation separation from *postnatal days* 2 -14 15 minutes Maternal separation from *postnatal days* 2- 14 1-hour restraint stress on postnatal days 49 +/- 1 day	↓ Basal glucose levels after RS T (CD3+)↓ 15 min of MS T (CD8 +) ↑ 15-min of MS B cells ↑ in both 15 and 3 h MS T (CD4+) ↓ in both 15 and 3 h MS Double positive (CD4+CD8+) NKT-like cells ↑ in 3 h MS (CD4-CD8-) NKT-like cells ↓ in 3 h MS CD8+ NKT-like cells ↓ in 3 h MS NK maturation ↑ in both 15 min and 3-h MS ↓NK degranulation and cytotoxity in 15-min and 3 h MS	Detailed NK immune cell population profile and its functionality by viSNE: an unbiased screening tool for flow cytometry data visualization.	PBMCs from individuals that had experienced ELA. 18-35 years of age 10-12 week old Wistar rats, 12 pups/dam adjusted

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CHAPTER TWO

Literature Review

Effects of Early Life Adversity on Mast Cells

Early life adversity occurring during a critical period of development such as the early years, have been suggested to influence the immune system and result in chronic inflammation later in life. For instance, early childhood adversities have been associated with high levels of inflammatory markers such as CRP, IL6 TNFα persisting throughout adulthood [1-6]. Also, numerous cohort studies have studied the associations between ELA and disease risk and found increased risk for cardiovascular diseases, type 2 diabetes, asthma, migraines and chronic pain, depression and psychiatric disorders [7-13], as well as increased severity of symptoms in patients with fibromyalgia [14] , premature mortality [15], and poorer prognosis in patients with lung and breast cancer [8, 16]. Mast cells are innate immune cells beneficial for the host and typically involved in normal physiological processes including vasodilation, angiogenesis, and pathogen clearance [17-20]. However, MCs have also been recognized for their negative role in exacerbating allergic and anaphylactic reactions [21], in addition MCs have also gained recognition as stress effector cells. This review focuses on the impacts of stress, particularly ELA on MCs and the involvement in stress-related immune and inflammatory disorders.

Mast Cell Background

Mast Cells are innate immune cells ubiquitously found in various tissues, skin, and mucosal sites, especially abundant at sites close to host-environment interfaces. Mast Cells were originally described by Paul Ehrlich in his 1878 doctoral thesis, as unique cells containing vast amounts of granules and having particular staining characteristics he named them “Mastzellen” meaning well-fed cells because they were

densely packed with granules [22, 23]. Mast cell progenitor cells (MCPc) (CD34+, CD13+, c-kit+, FcεRI-) are derived from the bone marrow myeloid progenitors cells (MMPc) (CD34+) [20, 24, 25]. Unlike other bone marrow derived cells, MCPc circulate the vasculature at low levels before differentiating into mature mast cells (MCs) and later localizing in peripheral tissues, adjacent to blood vessels, smooth muscle, nerves, and lymphatic glands where they exert their function. The stem cell factor (SCF) receptor c-*kit*, is pivotal for mast cell, development, differentiation, proliferation and migration. [26-29] and also critical growth factor is interleukin-3 (IL-3). SCF is produced by cells in the tissue and also produced by MCs. Mast cells are the only mature hematopoietic cell that expresses c-*kit* receptor [30]. Additional growth factors and mediators have been shown to promote survival, differentiation, growth and maturation of MCs [31-34]. It was shown that fibroblasts facilitated the development of IL3-dependent cultured MCs and could be co-cultured and maintained together [35, 36]. Another cytokine is IL4 which has been shown to synergize with SCF in maintaining MC survival, proliferation and IgE-dependent responses [37], other cytokines involved in MC survival and maturation are IL-9, IL-10, IL-33 and TGF-β and NGF [26].

After localizing mast cells live long and remain on tissues where they eventually will respond to different stimuli [38-40]. Mast cells are divided in subpopulations based on the tissue localization, species, and their capacity to synthesize and store various secretory granules which can be vitalized using cationic dyes. In mice, mast cells from skin, peritoneal cavity and connective tissue have different protease content compared to MC from mucosal sites [28, 41, 42]. Mucosal MCs are localized in mucosal sites such as gut mucosa and respiratory mucosa they contain relatively little heparin

proteoglycans in their granules corresponding in lower stored histamine concentrations. Connective tissues MCs are commonly found in connective tissues, such as skin and peritoneal cavity [22, 43, 44], their granules contain heparin proteoglycan and high levels of histamine. In humans, mast cells that contain tryptase as the major protease are similar to mouse mucosal mast cells both contain tryptase and chymase and are similar to mouse connective tissue mast cells. Mast cell populations present at a particular location are influenced by the tissue microenvironment and therefore are likely to change phenotype and thereby function. Mast cells are capable of releasing a variety of mediators in response to different stimuli including stress, these mediators released can induce changes in blood flow; epithelial permeability, cytokine release, and immune cell recruitment, all of these mediators are responsible for initiating, amplifying, and prolonging inflammation and subsequently leading to chronic diseases (**Fig. 2.2**). Mast cell plasticity is important, particularly during infections and during immunological or stress-induced challenges. [27, 43].During bacterial infections, MCs guarding the site of infection are capable of sensing and recognizing a wide range of bacterial pathogens and components thereby releasing the appropriate mediators such as TNFα [45, 46], IL6 [47], IL4 [48] and MC protease-6 [49] in a regulated manner into the surroundings recruiting other neutrophils and antigen presenting cells to the site of infection promoting bacterial clearance[50]. In a similar manner during stressful conditions MCs respond to neurogenic signals by communicating with sensory nerves through neuropeptides, histamine, serotonin, tryptase nerve growth factor and endothelin 1 [51-53]. MCs are highly involved in immunological processes; they can be activated by many mechanisms some of which

could be detrimental to the host, for example in allergies and inflammatory diseases such as IBS and IBD [54-58]. MCs can be activated through various receptors including, high-affinity IgE receptor also known as (FCεR1) and MAS-related GPR family member X2 (MRG PRX2). Different pathogens can also activate MC through pattern-recognition receptor (PRR's). Additionally, MC's possess various receptors for chemokines; complement components, cytokines, complementary receptors such as IgG multimeric receptors (FcγR) additionally several studies have shed light on possible mechanisms by which stress can activate MCs. Most of these studies propose that peripheral nerves release neuropeptides or hormones such as corticotropin-releasing factor, substance P, and neurotensin, and these are sensed through receptors MC. They are positioned adjacent to nerve endings [59-65]. MCs can become activated and degranulate in response to various external stimuli, including the classical allergen-specific activation, cross-linkage of the IgE receptor, which induces MCs to become activated degranulate in response to toxic substances, complement factors, cytokines, bacterial components, neuropeptides, and stress. Upon activation, MCs release various preformed granules such as histamine, serotonin, lysosomal hydrolases, proteases, tryptase and chymase, carboxypeptidase A3, growth necrosis factor (TNFa) and vascular endothelial growth factor (VEGF); also, MC activation leads to the de novo synthesis of many compounds including, lipid mediators, leukotrienes, prostaglandins, platelet-activating factor, and different cytokines and chemokines (**Fig 2.3**)

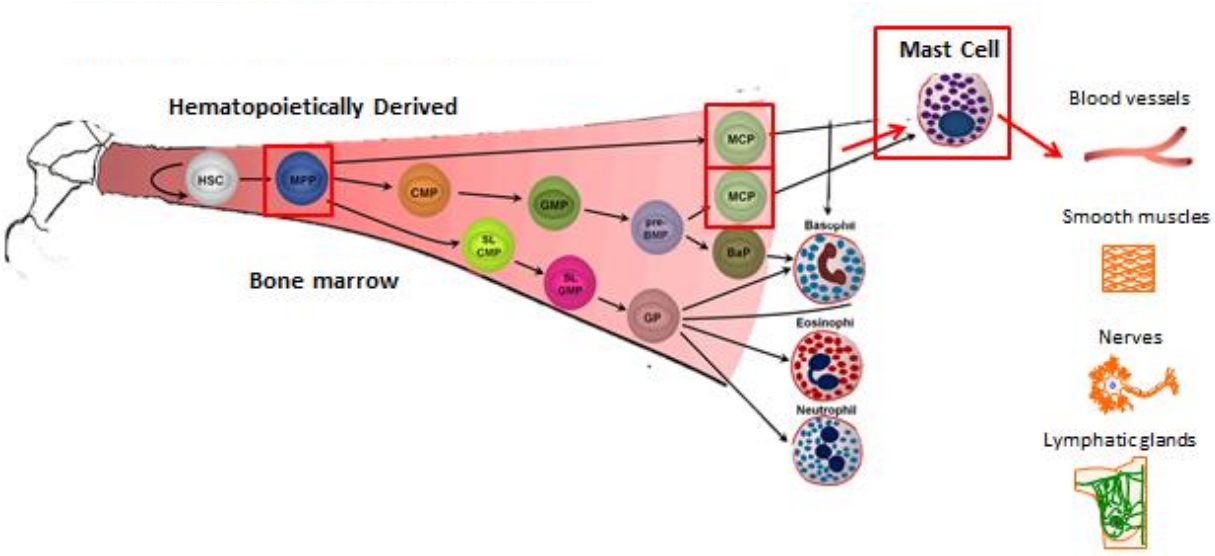

Figure 2. 1 Mast Cell Development and Location Adapted from "Mast cell progenitors: Origin, development and migration to tissue" by Dahlin J. and Hallgren J. 2015, *Molecular Immunology, 63(1): p. 9-17. Copyright © with permission 2014 The Authors by Elsevier Ltd.*

Mast cells originate from mast cell progenitor cells (MCPc) that are hematopoietically derived from the bone marrow from myeloid progenitors cells (MMPc) they circulate the vasculature at low levels before differentiating into mature mast cells (MCs) and later localizing in peripheral tissues. Mast cells are found near barriers, adjacent to blood vessels, smooth muscle, nerves, and lymphatic glands. Where they go and exert their function.

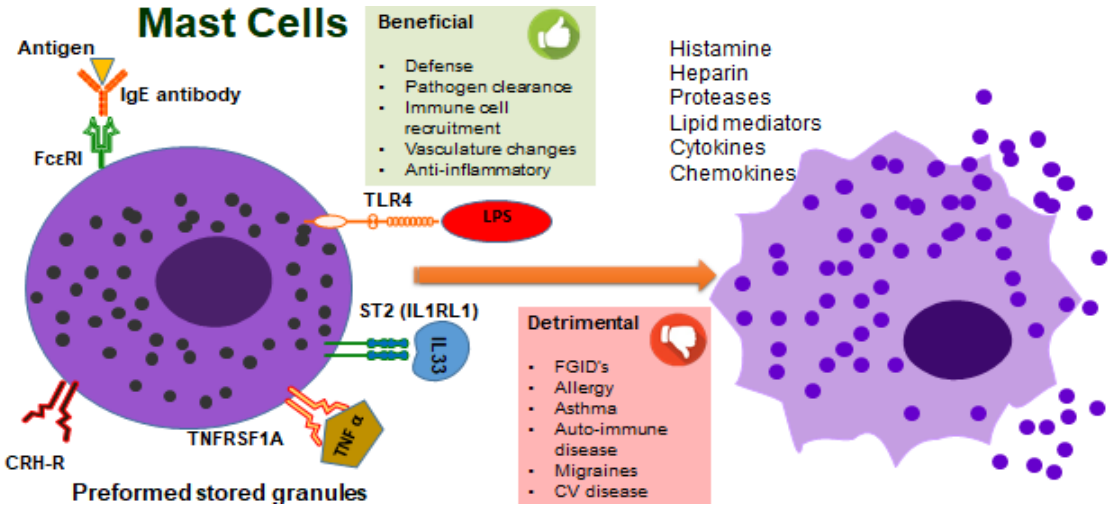

Figure 2. 2 Mast Cell Role in Health and Disease

Mast cells are beneficial and necessary for the host they are involved in different processes including defense and clearance of pathogen, vasculature changes and immune cell recruitment. Additionally, they are decorated with different receptors from which they can become activated. The most known are FceRI, the receptor for IgE, TLR 4 the receptor for LPS, ST2 the receptor for IL33 and there other various receptors from which mast cells can become activated like neuropeptides, inflammatory factors like complement and bacterial components. Upon activation they release different mediators and cytokines including histamine, heparin, proteases, lipid mediators, cytokines, and chemokines. Which are ultimately responsible of initiating, amplifying and enhancing inflammation.

Mast Cell Secretory Granule Biogenesis and Sorting

Mast cell granule biogenesis starts at the trans-Golgi, where small vesicles known as pro-granules are released and then fused together that later undergo a maturation process dependent on serglycin type proteoglycans in which a dense core is formed filling granules with bioactive amines, lysosome hydrolases and proteases. The

molecular mechanisms that regulate these processes are poorly understood, however, MC granules are structurally and functionally similar to vesicles of neuro-endocrine cells and neurons which have been widely studied. Mast cells contain various membrane associated proteins which partake in the biogenesis and exocytosis process. Puri et al 2008 showed granule heterogeneity in mast cells derived from the bone marrow of genetically altered mice lacking specific SNARE membrane fusion proteins where certain granules contained serotonin and cathepsin D and other granules contained TNF and histamine, where VAMP-8 deficient mice MCs exhibited defects in exocytosis. [66]. This demonstrated that MCs possess different subset of granules whose function is regulated by different SNARE proteins. Knowing the regulatory networks for each subset of granules will allow potential targeting of specific granules for therapeutic purposes. Also it has been shown that secretogranin III (SgIII) regulates the production of granules in MCs, as shown by an increase in MC granules of RBL-2H3 cells that overexpress SgIII [67]. Moreover, SgIII has been found to regulate granule load, and intensity, and length of inflammatory responses [67]. GTPase RAB5 has also been found to regulate granule size. [68]. Two central models have been proposed for the sorting of future granule components into granules. In the *sorting-by-entry model*, one example is the mannose 6- phosphate system (M6P) in which glycosylated proteins acquire M6P group that interacts with its receptor in the Golgi membrane, therefore only glycosylated proteins are selected for entry. In MCs each granule component has a sorting signal that interacts with a specific receptor in the *trans*-Golgi membrane. Notably, TNF sorting into MC granules has been found to be dependent on *N*-glycosylation, potentially linking the M6P system [69]. In the *sorting-by-retention* model

multiple granule components are initially enclosed in the immature granule and later maturation is achieved by the removal of selected components [70]. Synaptotagmin IX plays a role in the sorting of MC granules by segregating granules from the endocytic recycling compartment [71]. MC granules contain histamine, which is not only released when encountered with an allergen or toxic substance but it is also released upon tissue injury. It is known to promote vasodilation and bronchoconstriction [72]. Histamine enters granule through the vesicular monoamine transporter 2 (VMAT2)[73]. After biogenesis MC granules undergo maturation and are filled with mediators [74, 75]. Further studies are needed to shed light on the influence of early life induced-stress on MC granule biogenesis and heterogeneity.

Classic Mast Cell IgE Activation

Mast cells possess on their surface the receptor for IgE (FceRI). The FcεRI is composed of an IgE-binding α subunit, a β subunit, and two γ subunits of which contain immunoreceptor tyrosine-based activations motifs (ITAMs). When FceRI binds antigen, crosslinkage causes the LYN-dependent phosphorylation of ITAMs and activation of tyrosine- protein kinases (FYN, and SYN). Phosphorylation of adaptor proteins by these kinases induces the activation of phospholipase C. Subsequent production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) causes the release of calcium from the endoplasmic reticulum and activation of protein kinase C. Calcium release from the ER primes stromal interaction molecule 1 (STIM1) to open calcium operated channel ORAI1, which leads to extracellular calcium influx. Intracellular calcium and activation of protein kinase C activates the degranulation machinery forming microtubules and allowing granules to translocate to the plasma membrane. Later

coronin 1A and coronin 1B aids in the cortical actin depolymerization. Mast cells release the granules via a process of granule exocytosis which consists of extensive granule–granule and granule- plasma membrane fusion events, mediated by several soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), enabling MCs to release their contents effectively and efficiently [76, 77].

Alternative Non-IgE Mast Cell activation

Mast Cells are key players in the first response against pathogens; they are capable of responding to injured or contaminated tissue. Mast cells possess a number of pathogen-associated molecular patterns (PAMPs) pattern recognition receptors (PRRs). The most recognized PRRs are the toll-like receptors (TLRs). Mast Cells express TLR1-9, activation through these receptors does not induce a classical allergen degranulation but a release of chemokines, leukotrienes and cytokines including TNFa, IL-6, IL-5, IL-10, IL-1B, CCL1, CCL2 [78]. MC mediators are known to play a role in in enhancing and prolonging inflammation by activating dendritic cells [79, 80], NK cells [81], eosinophils [82], neutrophils and other nearby MCs [83-85]. Moreover, pro-inflammatory cytokines are also involved in regulating antigen presentation as well as activation of B cells. Toll like receptor 4 is the receptor for lipopolysaccharide (LPS), derived mainly from gram positive bacteria, engagement through this receptor needs associated co-receptors CD14 and MD-2 to activate the myeloid differentiation primary response 88 (MyD88) pathways. Once MyD88 activates this leads to activation of MAPK members ultimately inducing activation of transcription factors AP-1, NF-KB, and IRF-85 leading to the production of chemokines and cytokines [59, 86]. Another potent MC activator is IL-1 family member IL33. It is expresses in fibroblasts and epithelial

cells. It is already stored in nucleus of cells and released upon inflammation, injury or stress. IL-33 binds to ST2 also known as IL-1RL1. St2 is expressed in MCs and once activated it leads to a cascade of signaling triggering the activation of the MyD88-IRAK-dependenpt pathways leading to activation of NF-kB, Jun kinase and MAPK. Activation through ST2 receptor leads to the production of pro-inflammatory cytokines including IL-6, IL-13, IL-8, IL-4 and chemokines such as mast cell protease- (MCP-1 and MIP-1a) [43, 87]. While MCs' activating mechanisms are necessary for defense and repair, uncontrolled MC activation during stress can lead to the various symptoms involved in ELA-related diseases such as visceral hypersensitivity, abdominal pain, and neuroinflammation [88-93] and can long-term contribute to chronic inflammation. Thus, a balance is necessary to maintain ideal protective immune activation levels vs. overactive and detrimental inflammatory processes. Therefore, it is essential to understand how ELA influences MC activity and how it sets up later disease susceptibility.

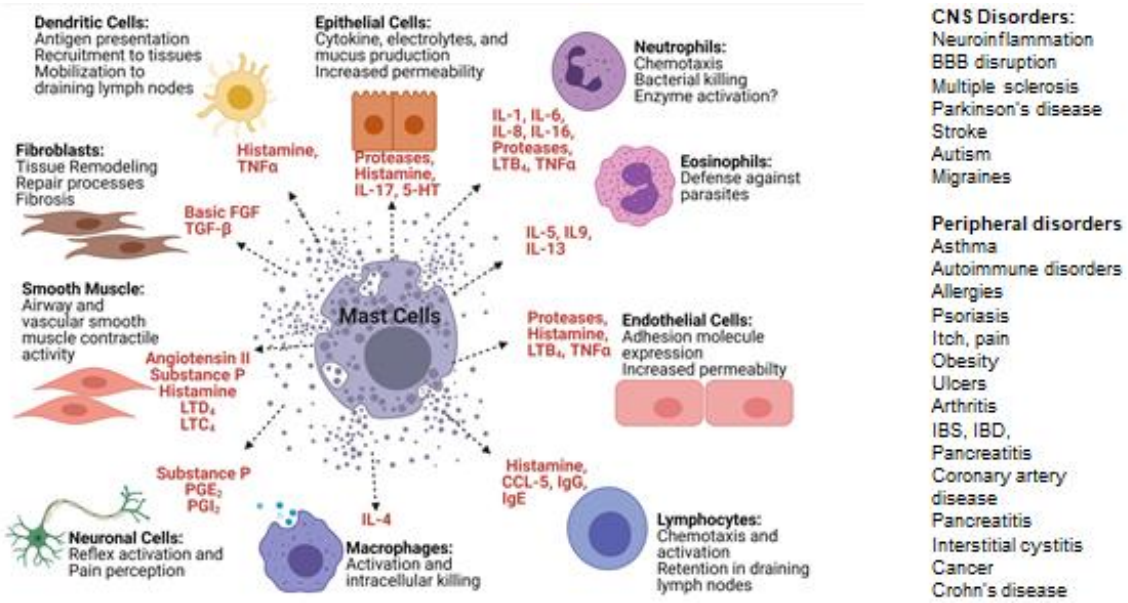

Figure 2.3 Mast Cell Contribution to Inflammation. Adapted from "Mast Cell Proteases and Inflammation" by Dai H. and Korthuis R. 2011, *Drug discovery today. Disease models, 2011. 8(1): p. 47-55. Copyright © with permission 2011 Elsevier Ltd. All rights reserved.*

Mast cells contribute to inflammation because they are able to communicate with other cells via mediator release and thereby are responsible of initiating, amplifying, enhancing and influencing Inflammation and also involved in the development of CNS associated diseases and peripheral disorders.

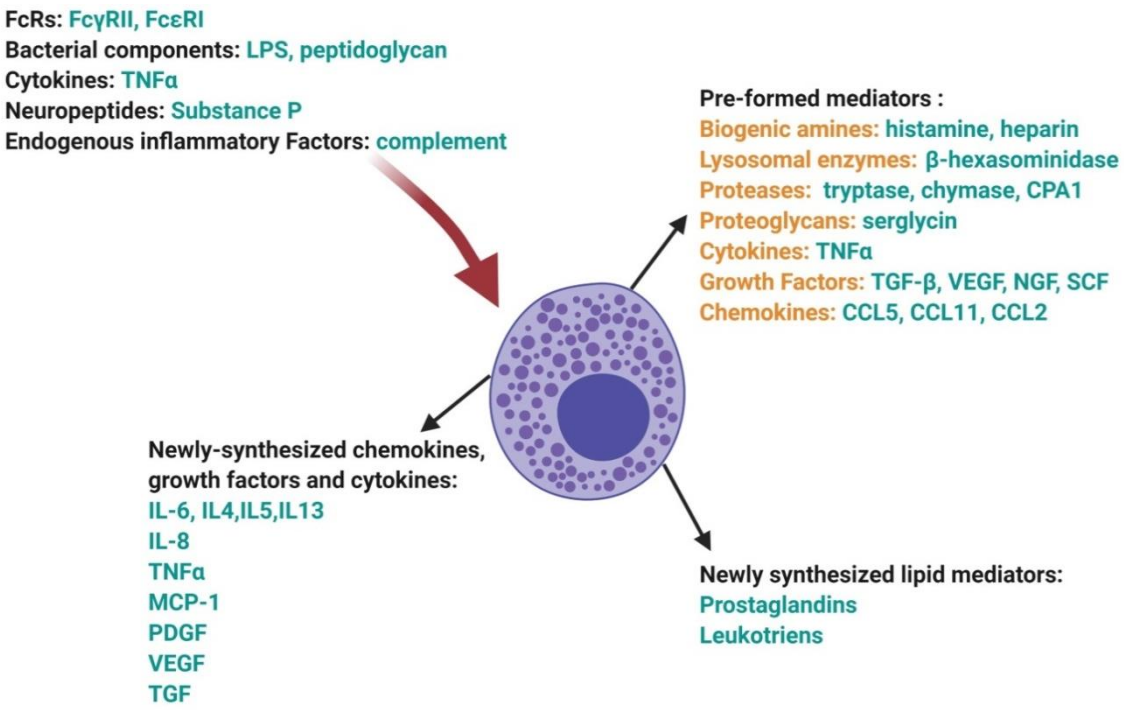

Figure 2. 4 Mast Cell Activators and Mediator Release

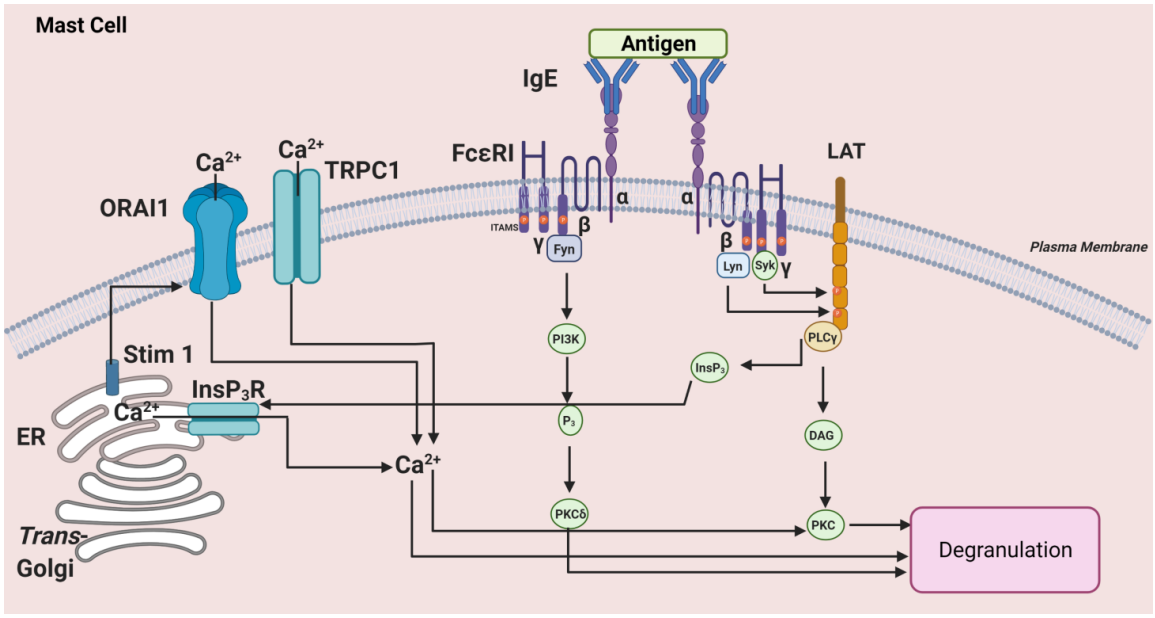

Figure 2. 5 Mast Cell Classic IgE Activation. Adapted from "Mast cell secretory granules: armed for battle" by Wernersson S. and Gunnar P. 2014, Nature Reviews Immunology, 2014. 14(7): p. 478-494. Copyright © with permission 2014 Nature Publishing Group, a division of Macmillan Publishers Limited. All rights reserved.

Effects of ELA on Mast Cells

Increasing evidence suggests that psychological stress triggers or aggravates the development of various, including metabolic, cardiac, and inflammatory diseases such as autoimmune diseases and multiple sclerosis [10, 94-97]. MCs are suspected of playing a role in the development and exacerbation of stress-induced diseases. This is largely because mast cells are filled with pre-formed mediators, and are strategically localized throughout the body near barriers and host environment interfaces and in proximity to neurons. Additionally, MCs are decorated with a large number of cellular receptors for communication with a range of cell types and mediators such as neurons, fibroblasts, and smooth muscle cells [98-101]. This property allows MCs to influence a wide-ranging functional activity throughout the body [102] and rapidly sense and create a stress response to mobilize and enhance an effective immune response by releasing pre-formed and neo-synthesized mediators, thereby alerting the immune system and impacting and coordinating multiple organ systems. Furthermore, MCs can release and responds to neurotransmitters, such as norepinephrine, acetylcholine and substance P which can regulate immune activity; conversely, neuroendocrine mediators such as CRH and alpha-melanocyte-stimulating hormone are known to regulate cytokine activity [102-104]. This reveals that the neuroendocrine system and the immune system are intimately interrelated and that they are capable of influencing each other.

Mast cells (MCs) have become recognized as important early immune effector cells in the stress response and stress-related pathophysiology. MCs have also been found to play a role in stress enhanced visceral perception and increased stress-induced colonic mucus secretion and colonic motility [20, 105, 106]. Mast cells are increased in numbers in models of early life adversity in multiple organ tissues.

Although there has been a lot of work focused on the brain and gastrointestinal tract additional studies in the bladder and peripheral tissues suggest that ELA might be affecting other organs (**Table 3**)**.**

Psychological stress has been shown to increase MC degranulation through MC receptor corticotropin-releasing factor receptor subtype 1 (CRF1) [55, 107] and downregulate MC degranulation through CRF2. Further, stress-induced gastric ulcer formations could be prevented using misoprostol an antiulcer prostaglandin which reduced MC destruction in gastric and duodenal tissue and mucosal histamine depletion, suggesting MCs could be key players in the release of stress-induced gastric histamine [108]. Further, fexofenadine, an antihistamine H1-receptor antagonist, reduced stress-induced visceral hypersensitivity in maternally separated adult rats subjected to later water avoidance stress. RBL-2H3 cells were used in this study to assess the possible MC stabilizing abilities of fexofenadine and results showed a decrease in the release of b-hexosaminidase in fexofenadine pre-treated RBL-2H3 cells [109]. Histamine is a MC mediator and has been known to act as a neurotransmitter and neuromodulator [110-112]. Histamine on the brain has been shown to increase after exposure to chronic stressors such as, exposure to cold, air blast, restraint, maternal separation chemicals exposure, foot shock, although it is still not known if it is MC mediated [113-118]. Also, histamine was shown to increase cytosolic Ca^{2+} levels of co-cultured submucosal neurons from rat's colon with RBL-2H3 cells a MC equivalent through H1R receptor. This highlights the importance of histamine and H1R in the communication between submucosal neurons and MCs. Furthermore that histamine

receptors on the epithelium and submucosal neurons permit MCs to interfere at different levels with various gastrointestinal functions [119, 120].

Animal models of multiple sclerosis correlated ELA and increased symptom severity with increased MCs in the brain [97]. Exposure to ELA was shown to contribute to the development of experimental autoimmune encephalitis (EAE) prompting downregulation of β-adrenergic receptors in bone marrow derived dendritic cells [121]. Similarly, increased nerve growth factor (NGF), a neurotrophic factor that plays a crucial role in neuronal plasticity and survival of immune cells, including MCs, was significantly increased in adult Lewis rats that have been previously exposed to maternal separation and neonatal handling, also observed was increased clinical signs to experimentally induced allergic encephalomyelitis (EAE) in adult life. Interestingly, a significant increase of MCs was observed in the hippocampus and the thalamus of adult rats [97]. Exposure to ELA has also been associated with increased MC in proximity to nerves in the ileum and colon mucosa of children with IBS, also found to correlate with intensity and frequency of abdominal pain [122]. In line, similarly Santos, Martinez and Wan showed stress-induced MC activation and hyperplasia in the intestine of IBS patients [123-125]. Moreover, McClain et al., 2020 found that ELA increased MC numbers in myenteric plexus and MCs were found to be in close proximity to enteric glia, promoting MC-glial interactions mainly through H1R. Additionally, exposure to maternal separation increased MC activation in the bladder and prostate [126-128]. MCs can regulate barrier function through proteases and degradation of tight junction and also MC secretory mediators such as histamine and prostaglandins can further stimulate enteric nerves

. Early weaning is a stressful and traumatic event for newborn pigs and thus, a good model to study ELA in pigs. Previous studies in our lab has demonstrated heightened MC activity, increased disease pathology including increased prevalence and severity of diarrhea, intestinal permeability, and increased tryptase levels in the ileum of early weaned pigs. Moreover, jejunal barrier dysfunction correlated with increased MC, and MC stabilizer cromoglycolate ameliorated barrier dysfunction and reduced chloride hypersecretion, on the other hand activation of MCs by compound 48/80 and CRF1 receptor, enhanced barrier dysfunction, in response to early life stress [129-132]. Together, these studies demonstrate the permanent capacity of maternal separation to induce immune-lasting changes in peripheral tissues, including MC number and distribution, highlighting MCs' vital role in stress-induced related disorders.

Moreover, psychological stress has been linked to heightened CRH levels in patients with mastocytosis, psoriasis and atopic dermatitis [133-135]. Crompton et al., 2003 demonstrated that CRH, which plays a role in directing the hypothalamic-pituitary-adrenal axis and the systemic response to stress caused vasodilation in human skin via mast cell-dependent pathways primarily mediated by histamine. In an early life adversity model of maternal separation Chen et al., 2021 demonstrated that ELA induced long-term visceral hypersensitivity along with increased MC activation and increased CRF+ cells in the PVN of adult rats, in addition, increased histamine, tryptase, IL-1B, IL6 and TNF-a release was also observed, the increased visceral hypersensitivity, increased pro-inflammatory cytokine levels, and CRF+ cells in the PVN that could be later blocked and ameliorated with intra-PVN injection of cromolyn, a known MC stabilizer. These results suggests that MC contribute to the proinflammatory cytokine and mediator

release in the PVN of early life stressed animals and that MC might be playing an important role in neuronal communication. Further studies are needed to elucidate the mechanism by which MC in the PVN and CRF neurons affect early life stress induced hypersensitivity. [136]. Additionally, our previous studies in the lab have also shown the long-term impact of ELA and secondary stress in GI MC number and activation [130, 137].

Further, ELA has been shown to disrupts MC and glia interactions; myenteric glia responds to histamine released by MCs, and H1 receptor antagonists blocked these responses [138]. Acute and chronic stress has also been described to exacerbate skin diseases like psoriasis and acne [139-141]. Interestingly, human skin expresses CRH [142].In the skin, MCs play an important role in the stress-induced cutaneous inflammation, MCs are located in the upper dermis near blood vessels and near neurons; they are involved in urticaria and the onset of psoriasis potentially communicating with adjacent sensory nerves via neuropeptide secretion [143-145]. Social isolation has been shown to reduce the number of MCs in the brain and increase histamine content in the hypothalamus, although there is still no explanation on how stress induces changes in mast cell number and mediator content. [146]. Restraint stress-induced 70% percent of degranulation in intracranial MCs and observed increased MC protease -1, stress-induced activation was eliminated by utilizing an antiserum to CRH. [64] Heightened MC number or increased MC activity has been found in ELA-associated diseases [97, 130, 136, 147] suggesting that MCs and MC mediators might be playing a role in MC playing a role in symptom severity.

While the literature from human studies and animal models demonstrate a significant impact of ELA on lifelong immune responses, the mechanisms driving these changes have yet to be fully elucidated.

Table 3. Animal studies establishing the effects of stress on mast cells

Reference	Type of Stress	Relevant findings	Animal/Sample information
(D'Costa et al, 2019)	*Immunological* Passive systemic anaphylaxis (PSA) *acute psychological stress* 1 hour and 3 hour restraint stress	MC CRF_2 negative global modulator of MC degranulation. CRF_2 knockout ↑ serum histamine levels. CRF_2 knockout ↑ PSA-induced anaphylactic responses CRF_2 knockout ↑ stress-induced colonic permeability CRF_2 knockout BMMC engraftments ↑ stress-induced colonic permeability CRF_2 negatively regulates Ca^{2+} signaling	*In-vitro* degranulation assays with BMMCs from 8-10 week old C57BL/6 wildtype $CRF_2^{+/+}$ and knockout $CRF_2^{-/-}$ mice and RBL-2H3 MCs overexpressed or silenced CRF_2 *In-vivo* MC responses and associated pathophysiology in: Passive systemic anaphylaxis (PSA) and acute psychological stress Measured in WT knock out mice and MC deficient mice engraftments Kit $^{W\text{-}sh/W\text{-}sh}$

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
Overman et al, (2012)	*Basal responses* Porcine ileum exposed to CRF.	Porcine ileum pretreated with CRF ↑ MC degranulation, MC tryptase and TNFα. Pre-treatment of ileum with MC stabilizing agent sodium cromolyn blocked CRF mediated degranulation and TNFα. CRF ↑ intestinal permeability via MC dependent release of TNFα and MC proteases TTX blocked CRF induced MC degranulation and increased intestinal permeability.	Female and male yorkshire x Hampshire cross-bred pigs of 6-8 weeks of age *Ex-vivo* Ussing chambers Ileum harvested pretreated with CRF and CRF antagonist, Astressin B Neuronal blocker TTX prior to CRF exposure
Reimann et al, 1987)	*Stress-induced ulcer* (cold chamber for 5 hours) *Pharmacological* stress Histamine, aspirin, and ethanol induced-gastric ulcers	All forms of stress induced a ↓ in MC count and ↓ in mucosal histamine in gastric and duodenal tissue due to cellular destruction and damage. Suggesting major source of gastric histamine must be from MCs. Misoprostol attenuated stress-induced gastric ulcer formation, prevented reduction in MC count and mucosal depletion of histamine.	Stomachs and intestinal tissue from male Charles river guinea pigs subjected to ulcer tests Pharmacologically induced ulcers Misoprostol an antiulcer prostaglandin used as analog.

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
(Stanisor et al, 2013)	2-hit stress Neonatal Maternal separation 180-min/day Postnatal day 2-14 + 1- hours water avoidance stress	In NMS Long-Evans rats MC degranulation plays important role in the development of stress-induced visceral hypersensitivity and loss of barrier integrity Water avoidance ↑ hypersensitivity to distension in NMS rats, response blocked with fexofenadine an antihistamine H1-receptor antagonist and ebastine a second-generation antihistamine ↓ In β-hexosaminidase in fexofenadine pre-treated RBL-2H3 cells	*In-vivo* Long-Evans rats Visceromotor response to colonic distention Treatment with fexofenadine and ebastine *In-vitro MC degranulation assays* RBL-2H3 cells treated or not with fexofenadine
(Manni et al, 1998)	2-hit Neonatal maternal separation(2-hours daily) + Gentling every 5 minutes pups were placed in palm of hand and caressed on back and stomach for 15 min. (*Postanatal day 0-20*)	Differences in MC numbers between NMS and control groups are significant. NMS ↓ number of MCs in the hippocampus and thalamus of rats at 3 weeks and ↑ MCs at 60 days of age NMS ↓ brain nerve growth factor in hippocampus at 9 and 19 days of age; ↑ EAE clinical signs in rats separated from mothers	Lewis rats Experimentally induced allergic encephalomyelitis (EAE) induction

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
(McClain et al 2020)	2-hit stress Neonatal maternal separation + early weaning 3-hours *Postnatal day 1-16* early weaning at *PD17*	ELA ↑ MC numbers (tdTomato+ mast cells) in myenteric plexus. Exposing enteric glia to supernatants from IgE-DNP stimulated MCs evoked an ↑ in e Ca^{2+} responses of enteric glia. Exposing enteric glia to supernatants from IgE-DNP stimulated NMS-MCs evoked an ↓ Ca^{2+} responses of enteric glia suggesting ELA causes changes in MC that affect production of mediators that activate enteric glia. MCs are in close proximity to enteric glia, promoting MC-glia interactions through H1R.	$Mcpt5^{Cre}$;GC*a*MP5*g-td*T mice to genetically tag mast cells as to accurately study their association with enteric glia Female and male C57BL/6 wild-type I*n-vitro* BMMCs were harvested from femurs of 10- wk-old female and male C57BL/6 mice and cultured IgE-DNP stimulations
(Pohl et al, 2017)	Porcine early weaning stress model of ELA Piglets weaned from sow at 15 days of age (early weaning) or 28 days of age (Late Weaning)	Juvenile and adult early weaned pigs ↑diarrhea, ↑intestinal permeability and ↑MC numbers. ↑MC co-localization with neuronal ganglia in early weaned pigs. Female pigs ↑ ileal MC tryptase release upon activation.	Yorkshire- duroc cross, female and Male- C piglets (castrated at 9 days of age). Functional diarrhea, ileal permeability, MC activity MC relationship with enteric glia

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
(Mackey et al, 2016)	*Immunological stress* (2 h of IgE-mediated passive systemic anaphylaxis (PSA)) or *Psychological stress* (1 h of restraint stress (RS))	Sexually dimorphic responses Female ↑ PSA-induced pathophysiology, ↑increased serum histamine levels Female ↑Intestinal permeability and serum histamine after psychological stress.	C57BL/6 male and female mice BMMCs were harvested from femurs of 10- wk-old female and male C57BL/6 mice and cultured IgE-DNP stimulations
Moeser et al, 2007)	Porcine early weaning stress model of ELA Piglets weaned from sow at 15 days of age (early weaning) or 28 days of age (Late Weaning)	Early weaning ↓ trans- epithelial electrical resistance and ↑ mucosal permeability to [^{3}H] mannitol in jejunum and colon. Early weaning ↑ mucosal expression of CRF receptor 1 protein. Early weaning ↑ tissue MC degranulation. Pretreatment of piglets prior to weaning with cromolyn a mast cell stabilizer eliminated the early-weaning-Induced intestinal barrier disturbances.	Female and male Yorkshire crossbred piglets

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
Pierce 2018	Neonatal Maternal separation for 3 h/day *Postnatal Day 1–21*	NMS ↑ bladder sensitivity and ↑MC degranulation. NMS ↓ corticotropin-releasing factor receptor 1 (CRF1) and glucocorticoid receptor mRNA levels in the hippocampus.	Female C57Bl/6 mice Urinary bladder distension (UBD) at 8- weeks of age
(Fuentes et al, 2018)	2-hit stress Neonatal maternal separation (NMS) was performed for 3 h a day *Postnatal day 1 to 21* + 1-h water avoidance stress	NMS result in neurogenic inflammation and hypersensitivity in urogenital organs. NMS ↑ Perigenital sensitivity and micturition, this was enhanced after WAS NMS ↑MC degranulation in bladder and prostate this was also enhanced after WAS NMS ↑ Basal fecal pellet output; Bladder CRF^1 protein expression ↑ by NMS/WAS interaction; Prostatic CRF^1 protein ↓ in NMS–WAS compared to Baseline	Male C56Bl/6 mice

Table 3 (cont'd)

Reference	Type of Stress	Relevant findings	Animal/Sample information
(Smith et al, 2009)	Porcine early weaning stress model of ELA	Early weaning (15- to 21-day weaning age) ↑ impairment of intestinal function. Jejunal barrier dysfunction associated with ↑mucosal MCs. Mast cell stabilizer sodium cromoglycolate diminished early wean stress- induced pathophysiology. MC activation ex-vivo with c48/80 and CRF induced barrier dysfunction and was later abolished with MC protease inhibitors.	Female and male Yorkshire cross-bred piglets Piglets were assigned to one of five weaning age groups: weaning at 15, 18, 21, 23, or 28 days of age Mast cell activation *ex-vivo*
(Chen et al., 2021)	Neonatal maternal separation 6-h/daily *Postnatal day 2 -15*	NMS ↑ visceral hypersensitivity NMS ↑ MC activation and increased CRF+ cells in the hypothalamic paraventricular nucleus (PVN) of adult rats. NMS ↑ histamine, tryptase, IL-1B, IL6 and TNF-a release Increased visceral hypersensitivity, increased pro-inflammatory cytokine levels, and CRF+ cells in the PVN were later blocked and ameliorated with intra-PVN injection of cromolyn, a known MC stabilizer	Sprague-Dawley rats and mast cell deficient $Kit^{W-sh/W-sh}$

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CHAPTER THREE

The impact of Early Life Adversity on MC Activation and Mediator Content and Disease Susceptibility in Adulthood

ABSTRACT

Exposure to early life adversity (ELA) is a significant risk factor in later life susceptibility to inflammatory disorders, including allergy, asthma and chronic pain disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Mast cells (MCs) are innate immune cells known to play central roles in GI functional and inflammatory diseases and have been linked in the pathophysiology of stress-induced diseases. The mechanistic link between ELA and later life MC disease is poorly defined. The aim of this study was to investigate whether ELA impacts long-term MC activity and MC mediator content and additionally, determine whether ELA increased adult MC disease susceptibility. Female and male C57BL/6 mice pups were either raised under standard protocols (normal handling; NH) or subjected to 3 h periods of neonatal maternal separation on postnatal d 1-18 and early weaning (NMSEW). At 10 wks of age, histamine content from tissue-resident MCs from the peritoneal cavity of NH and NMSEW mice (pMC's) was measured. Additionally, to evaluate adult MC disease susceptibility, NH and NMSEW mice were exposed to two well-known and established MC disease models, chronic psychological stress (isolation and 2 – 4 h of restraint stress for 7 d), and IgE-mediated passive systemic anaphylaxis (PSA). For chronic stress serum was analyzed for histamine levels and mesenteric windows from jejunum were collected to assess tissue MC degranulation. Stool pellets were collected as an indicator of GI motility and FITC dextran gavages were performed to evaluate GI permeability. At baseline, NMSEW mice exhibited higher ($P < 0.05$) histamine levels (by 2 fold) and MC degranulation ($P = 0.01$) compared to NH controls. During chronic stress, NMS mice exhibited greater ($P < 0.01$) histamine levels (by 1.32 fold) and MC

degranulation ($P < 0.01$) compared with NMS mice. NMSEW mice exhibited higher stool pellet output ($P = 0.01$) following chronic stress compared to NH, indicating enhanced stress induced GI motility in NMS mice. Additionally NMSEW mice showed in an increase in intestinal permeability. We additionally subjected mice to adult immunological stress (PSA), briefly, mice were sensitized with anti IgE-DNP and antigen was injected a day later to induce anaphylaxis. Clinical scores and body temperature were measured as well as serum histamine and to measure MC activity. NMSEW mice exhibited increased PSA-induced hypothermia in a dose depended manner. NMSEW exhibited increased clinical scores compared to controls in both doses at 15 minutes post-injection. Greater histamine content was identified in NMSEW pMC's compared to NH pMC's. Results are in line with the previous MC hyperactivity seen in tissue resident MCs of mesenteries and higher serum histamine. Together these studies demonstrate that ELA induces lasting changes in MC hyperactivity and mediator content in addition to vulnerability to later life stress and clinical GI dysfunction, with females more at increased risk. Future research will be aimed at elucidating the mechanisms through which ELA programs MC development and function and its role in exacerbating later life GI and allergic disease models.

Introduction

Exposure to ELA is a major risk factor in the onset and exacerbation of inflammatory disorders later in life. Chronic stress disturbs the stress response system, compromising the negative feedback loop and preventing the return stress hormones to basal unstressed conditions. This in turn, can influence regulation of the immune system and increase inflammatory diseases later in life. ELA has been shown to induce increased pro-inflammatory cytokines and humoral cell infiltration and later susceptibility to inflammatory diseases including allergies when exposed to novel antigens [1-4]. Patients with a previous history of ELA associated disease display, increased symptom severity, increased epithelial permeability, increased susceptibility to stress and particularly, increased MC number or MC activation. Therefore, MCs have become recognized as important early immune effector cells in the stress response and stress-related pathophysiology [5-7]. MCs are capable of mediating brain-gut communications through CRH activation and neuronal communications [8, 9]. Psychological stress has been shown to increase MC activation through CRF1 receptor [10]. Furthermore, MC have been found to be key players in the development of stress-induced visceral pain and increased colonic motility [11, 12]. MCs are uniquely positioned to drive ELA-mediated inflammatory responses since they are distributed throughout the body and highly activated in response to physiological stress. Moreover, restraint stress has been shown to induce 70% of MC degranulation with increased MC protease in intracranial MCs. Furthermore, MCs are capable of releasing copious amounts of mediators in response to different receptors on their surface that can induce changes in blood flow, epithelial permeability, cytokine release, and immune cell trafficking; all of these

mediators and cytokines are responsible for initiating, amplifying, and prolonging inflammation and subsequently leading to chronic diseases.

Rationale: MCs play an important role in protecting the host against pathogens, but when MC become overactive or increased in number they can be detrimental. MCs have been found to be hyperactive or increased in number in a lot of ELA-associated diseases. Since, MCs rapidly degranulate in response to stress we therefore developed the working hypothesis that ELA increased MC hyperactivity and altered MC phenotype in adulthood, for this we validated an ELA model of neonatal maternal separation plus early weaning (NMSEW) in C56BL/6 mice that we can use this later to evaluate MC phenotype and activity. To further demonstrate MC hyperactivity, and MC disease susceptibility we modified our previous NMS model by adding the early weaning component to buffer any confounding effects of maternal care.[13] In our study, we investigate the influence of early life adversity on adult MC hyperactivity and mediator content by utilizing our ELA model (NMSEW) with the rationale of better understanding the impact early life stressors on adult MC activity. Here, we demonstrate that mice exposed to ELA exhibit increased MC activity into adulthood compared to controls. Moreover, that ELA affects MC number, phenotype and activation, increasing basal serum histamine, and increased histamine content in tissue MCs. We later demonstrated that ELA predisposed mice to increase MC activation following adult chronic psychological stress and immunological stress.

Methods

Ethics Statement

All experimental procedures were reviewed and approved by Michigan State University's Institutional Animal Care and Use Committee.

Restraint Stress

Female and male mice (10 weeks old) were subjected to restraint stress (RS) by placing them in an individual clear well ventilated 50-ml plastic conical tubes for 15 min); control mice (non-stressed) of each sex remained in their original home cages without food and water to avoid confounding effects of water or feed intake during the test period. Following RS, stressed and control mice were immediately euthanized by CO2 inhalation, and serum was collected to evaluate histamine concentration by competitive histamine ELISA (Oxford Biomedical Sciences, Rochester Hills, MI). Following euthanasia, mesenteric windows from the jejunum were collected and stained with toluidine blue (1% pH 1) to assess and tissue MC degranulation. Five high-power fields (×10 magnification) of mesentery from mice were randomly chosen, and MCs were counted and evaluated for degranulation in a blinded fashion. Fecal pellets were collected and counted as an indirect clinical measurement of GI motility.

Animal Care and Neonatal Maternal Separation plus Early Weaning (NMSEW)

C57BL/6 pregnant nulliparous dams (The Jackson Laboratory, Bar Harbor, ME) were isolated a few days before giving birth. The day the pups were first observed was determined as postnatal day 0 (PD0). At postnatal day (PD1) female and male C57BL/6 wildtype mice pups were randomly assigned to two groups: 1) Normal handled (NHLW); raised under standard protocols undisturbed and weaned at PD28 or 2)

Neonatal maternal separation (NMSEW); subjected to 3 hour periods of neonatal maternal separation (9:00AM-12:00AM) on PD1-16 plus early weaning at PD17. Pups in NH group remained undisturbed with their mother in their home cages during the entire NMS period and received no special handling other than that necessary to change their bedding, water and food. Pups in NMS group were separated daily from their mother for 3 hours and placed in a separate cage in individual cups containing small amounts of their own bedding. Meanwhile NMS mother was placed in a separate clean cage with food and water during the separation. After the 3 h of separation, the mother and pups returned to their original cage and remained untouched until next day of separation. After weaning all mice were cage with the same littermates/treatment containing no more than 4 mice per cage. All mice were maintained under specific pathogen free conditions in facilities accredited by the Association for Assessment and Accreditation for Laboratory Care (AAALAC) International. Animals were kept in a 12-h light-dark cycle at a constant temperature of 21±2℃ and relative humidity of 50-60% with *ad libitum* access to water and standard commercial rodent chow diet. All experiments were approved by the Animal Care Committee of Michigan State University and conducted accordance with the guidelines of the Care and Use of Laboratory Animals. Following euthanasia, ileum and colon samples were collected from pigs at 20 weeks and flash frozen on liquid nitrogen and stored at −80°C. Pups from first parity dams (C57BL/6) were either raised under standard protocols (normal handling; NH) or subjected to 3 h periods of NMS daily on postnatal d 1-16 plus early weaning at 17 d of age. At 10 weeks of age, adult mice were euthanized and serum was collected to

determine histamine levels and mesenteric windows from the jejunum were collected and stained with toluidine blue to evaluate tissue MC number and activation n.

Mast Cell Staining and Counting

Mast Cells were stained with 1% toluidine blue solution pH 1 for 30 minutes to detect mast cell degranulation using FIJI (Image J) (U.S. NIH, Bethesda, MD, USA)] 10 high-power fields per subject (x 10 magnification).

Fecal Pellet output

Restraint Stress accelerates colonic transit. Immediately after restraint stress the total number of excreted feces was counted.

FITC

Mice were fasted for 3hrs then gavage with FITC Dextran (450mg / 1g mouse) (Sigma, St. Louis, MO), Mice were bled 4 hrs. After gavage by submandibular stick with a 5mm lancet for later measurement of FITC Dextran by fluorometer with Synergy H1 plate reader (Biotek) with excitation at 485nm and emission at 528nm.

Passive Systemic Anaphylaxis (PSA)

NH and NMSEW mice were sensitized with IgE monoclonal Anti-Dinitrophenyl (0.5 µg/gram of mouse IgE anti-DNP) administered in 100µl PBS via the i.p. route. Mice were monitored for 15 minutes to ensure there is no bleeding or trauma associated with the I.P. injection site and that the mice return to their normal behavior once placed back in their cage. Twenty-four hours later mice were injected with 2 doses a low dose of 50 ug or a high dose of 500 µg of 2, 4-dinitrophenyl-human serum via the i.p. route. Clinical scores and rectal temperatures were monitored for 30 min utilizing the TH-5 Thermalert

monitoring thermometer with a rectal probe suitable for mice (Physitemp, Clifton, NJ, lubricant and lidocaine was applied to the probe before each use. Clinical scores verified the following symptoms associated with a anaphylactic response, scratching, itchiness, puffiness, swollen eyes/ face, reduced activity, labored breathing, increased respiration, tremors, inability to right itself, death. Scores were recorded and analyzed in a blinded fashion.

Isolation and Purification of Peritoneal Mast Cells (PMCs)

Peritoneal cells were harvested from 10 week old female and male NH, NMSEW C57BL/6 mice by performing peritoneal lavage with 10 ml of Hank's Balanced Salt Solution modified (1×) without phenol red and without calcium or magnesium salts, supplemented with EDTA (1 mM) to prevent mast cell degranulation. Removal of red blood cells was performed by adding 1 ml of 0.8% NH4Cl several times until a clear pellet was visualized. Subsequently, peritoneal mast cells (pMC's) were isolated using a 70% Percoll gradient. Purity of separation of pMC's was confirmed to be >95% by staining with toluidine blue. Count and viability of the cells >95% were assessed by trypan blue dye exclusion test in an automated Cell Counter (LUNA™, San Mateo, CA) equal number of cells was calculated and were lysed using radioimmunoprecipitation (RIPA) buffer supplemented with phosphatase and protease inhibitors, followed by sonication (Sonic Dismembrator Model 100, Fisher Scientific), for later histamine measurement.

Body Weight

Body weights were measured before euthanizing animals to compare weight gain of normal handled and neonatally stressed animals.

Statistics

Data were analyzed utilizing GraphPad Prism version 6 for Windows, (GraphPad Software, San Diego, CA, USA) Two- way ANOVA with post hoc Fisher's Least Significant Difference test where appropriate to determine effects of weaning, sex, or interaction and any specific difference between groups. Two-way repeated measures ANOVA was used to evaluate rectal temperature and clinical scoring in passive systemic anaphylaxis (PSA) model and LPS challenge; One-way ANOVA was used for comparison of more than two groups, followed by Tukey's multiple comparison tests. Unpaired Student's t test or Mann-Whitney U test as appropriate was applied to compare differences between two groups. Data are represented as the mean ± standard error of the mean (SEM).

Euthanasia and Sample Collection

At 10 weeks of age mice euthanized by CO2 inhalation, and the mesentery of the jejunum was collected and stained with toluidine blue (1%, pH 1) to evaluate tissue MC activation. Serum was collected for histamine and McP-1 measurements blood was collected at 30min. Histamine concentration by competitive histamine ELISA (Oxford Bio medical Research, Rochester Hills, MI) and mMcp-1 (LifeSpan BioSciences Seattle, Washington) according to the manufacturer's instructions.

Results

Mice Exposed to ELA (NMS+EW) exhibit Increased Mast Cell Activity into Adulthood

To start evaluating the influence of ELA on mast cell activation, we developed and validated an ELA model of neonatal maternal separation plus early weaning (NMSEW). Female and male C57BL/6 wildtype mice pups were randomly assigned to two groups:

1) Normal handled (NH); raised under standard protocols undisturbed and weaned at PD28 or 2) Neonatal maternal separation (NMS+EW); subjected to 3 hour periods of neonatal maternal separation (9:00 AM-12:00 AM) on PD1-16 plus early weaning at PD17. Pups in the NH group remained undisturbed with their mother in their home cages during the entire NMS period and received no special handling other than that necessary to change their bedding, water, and food. Pups in the NMS group were separated daily from their mother for 3 hours and placed in a separate cage in individual cups containing small amounts of bedding. Meanwhile, NMS's pup's mother was placed in a separate clean cage with food and water during the separation. After the 3 h of separation, the mother and pups returned to their original cage and remained untouched until the next day of separation. After weaning, all mice were cage with the same littermates/treatment containing no more than 4 mice per cage. All mice were maintained under specific pathogen-free conditions in facilities accredited by the Association for Assessment and Accreditation for Laboratory Care (AAALAC) International. Animals were kept in a 12-h light-dark cycle at a constant temperature of 21±2℃ and relative humidity of 50-60% with ad libitum access to water and standard commercial rodent chow diet. All experiments were approved by the Animal Care Committee of Michigan State University and conducted according to the Care and Use of Laboratory Animals guidelines. At 10 weeks of age, samples were collected to evaluate MC activation.

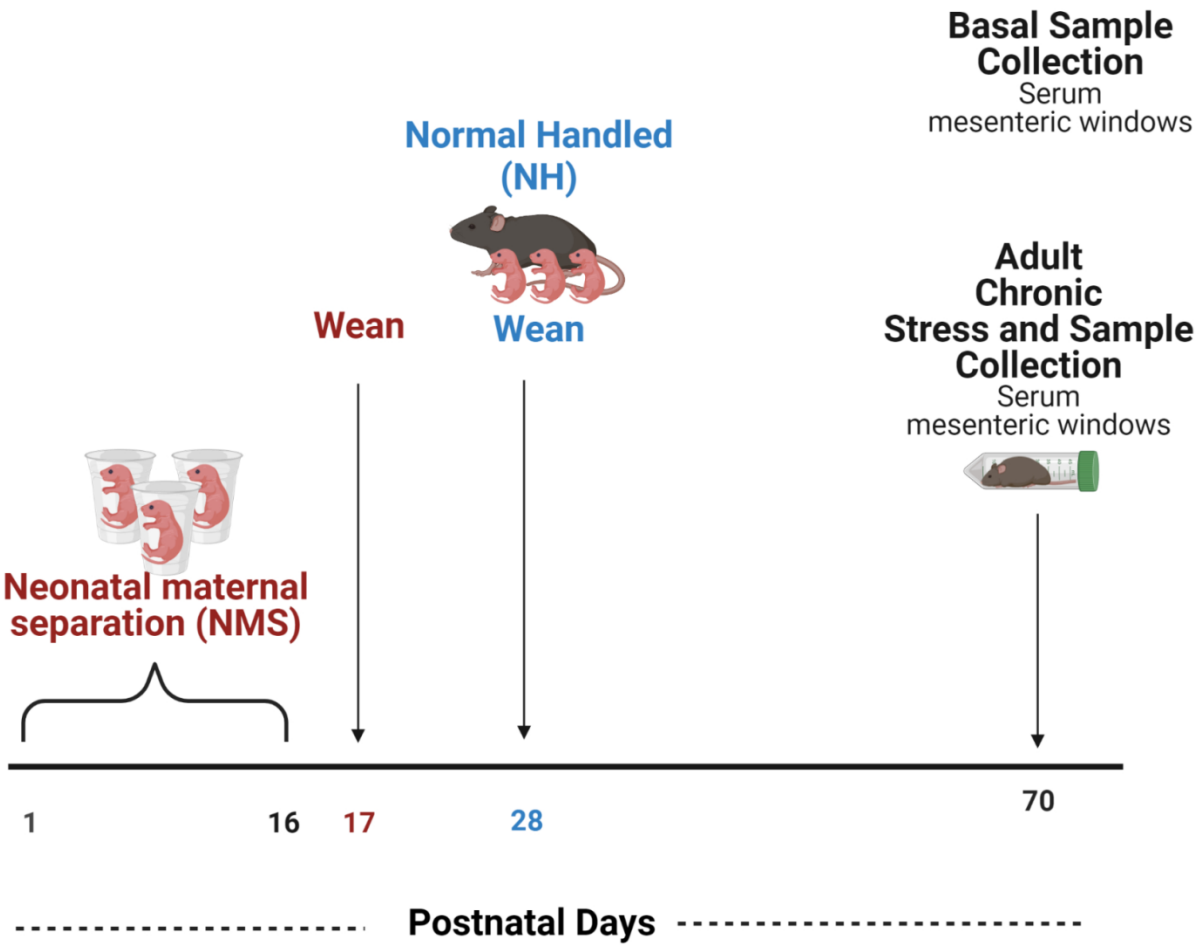


Figure 3.1 Early Life Adversity Model of Neonatal Maternal Separation plus Early Weaning.

To evaluate if ELA affected MC activity in adulthood, we assessed serum histamine levels from both our groups, control (NH) and NMS+EW, and evaluated GI MC activation by staining mesentery from jejunum with toluidine blue. We observed increased serum histamine levels in NMSEW mice (~2 fold higher, $P<0.05$) as well as increased mast cell activation in mesenteric windows of jejunum (~2 fold higher, $P<0.0001$) also, increased MC number $P<0.05$ compared to NH controls. **(Fig 3.2)**

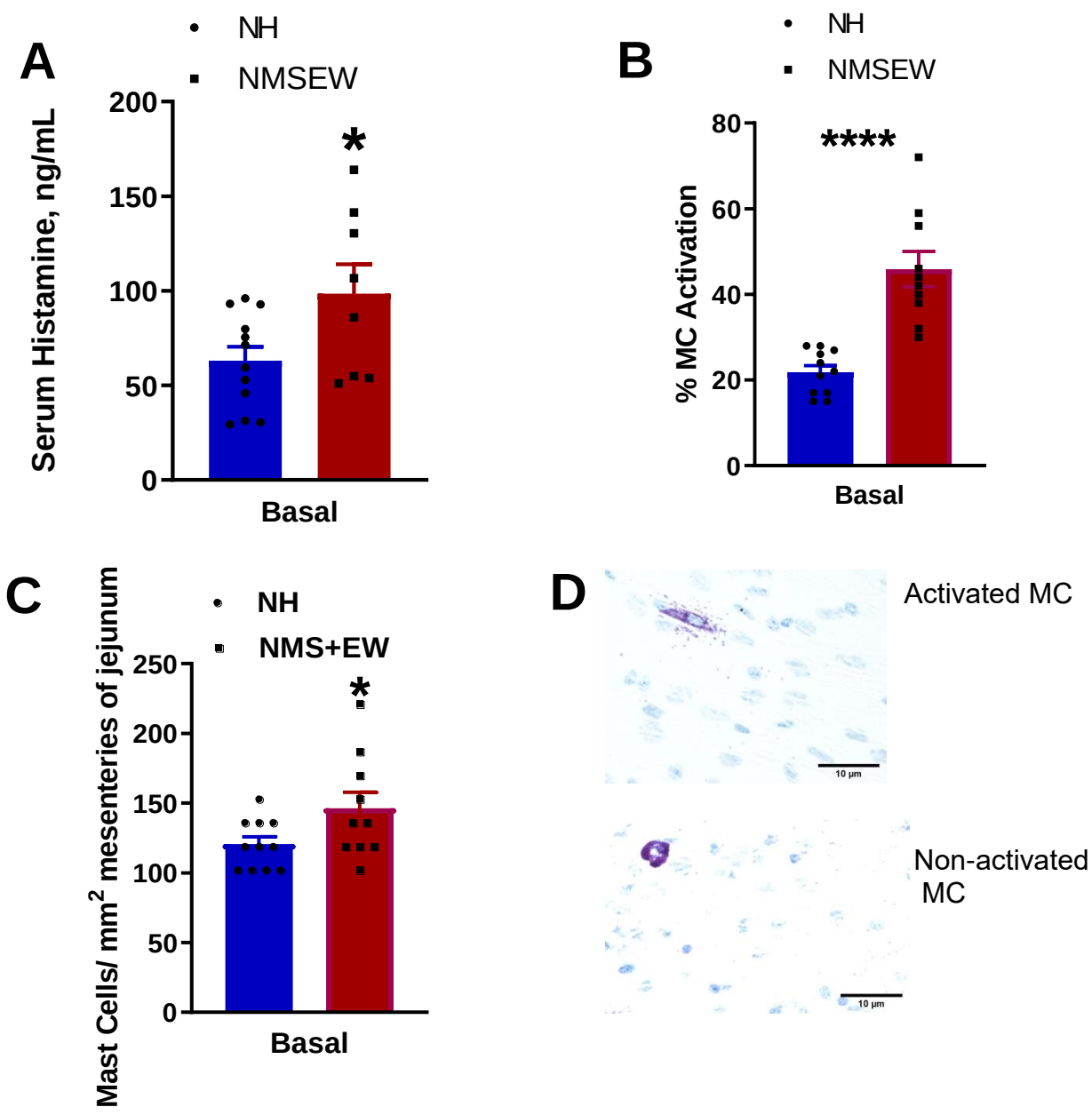


Figure 3.2 Impact of ELA on adult mast cell activation.

(A) Basal serum histamine levels females and males combined. **(B)** Percentage of MC activation in mesenteric windows from jejunum females and males combined **(C)** MC numbers in mesenteric windows from jejunum **(D)** Graphic representation of the scoring system utilized to assess activated MC and inactivated MC. GraphPad Prism 8.1.0,

Figure 3.2 (cont'd)

values represent mean ± SEM. Statistical analysis used, unpaired t-test, n = 10-12 mice per group, 5-6 per sex.

Early Life Adversity Predisposes Mice to Increased MC Activation Following Psychological Stress

To investigate if NMSEW mice and NH displayed differences in MC activation after a second-hit stress we subjected mice to a non-IgE- MC dependent model of chronic physiological stress. We assessed MC responses by measuring histamine levels of NH and NMSEW mice, for this For this, 10 weeks old were subjected to restraint stress (RS) by placing them in an individual clear well ventilated 50-ml plastic conical tubes, for 15 min); control mice (non-stressed) of each sex remained in their original home cages without food and water to avoid confounding effects of water or feed intake during the test period. For RS we evaluated intestinal permeability and fecal pellet count as a measure of GI motility, since previous studies have found GI alterations in restrained stressed animals. Serum histamine levels, mast cell protease--1 and mesenteries from jejunum stained with T-blue were evaluated to assess MC activation. Toluidine blue staining of jejunal mesenteries confirmed that RS lead to a higher MC activation. Serum histamine levels were evaluated as well as GI mast cell activation. We observed increased serum histamine levels ($P < 0.05$), ~2 fold in NMS+EW mice following chronic stress as well as increased GI mast cell activation ($P < 0.01$), GI motility ($P < 0.05$) and intestinal permeability ($P< 0.05$). No differences were observed in MC protease-1, but may be due to insufficient powering as it seems there is trend toward significance, with

females exacerbating this response. No sex differences were observed for mMcp-1 although this may be due to insufficient powering of the study. **(Fig 3.4)**

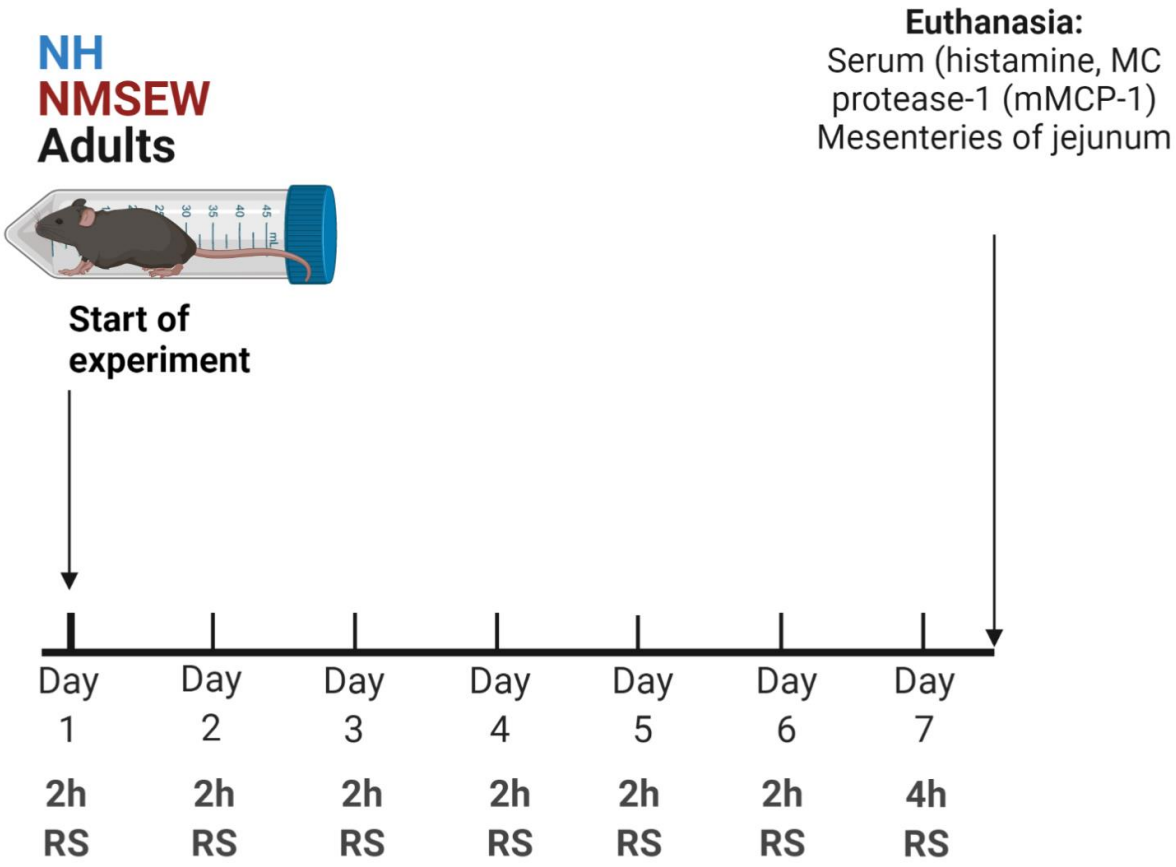


Figure 3.3 Restraint Stress Protocol.

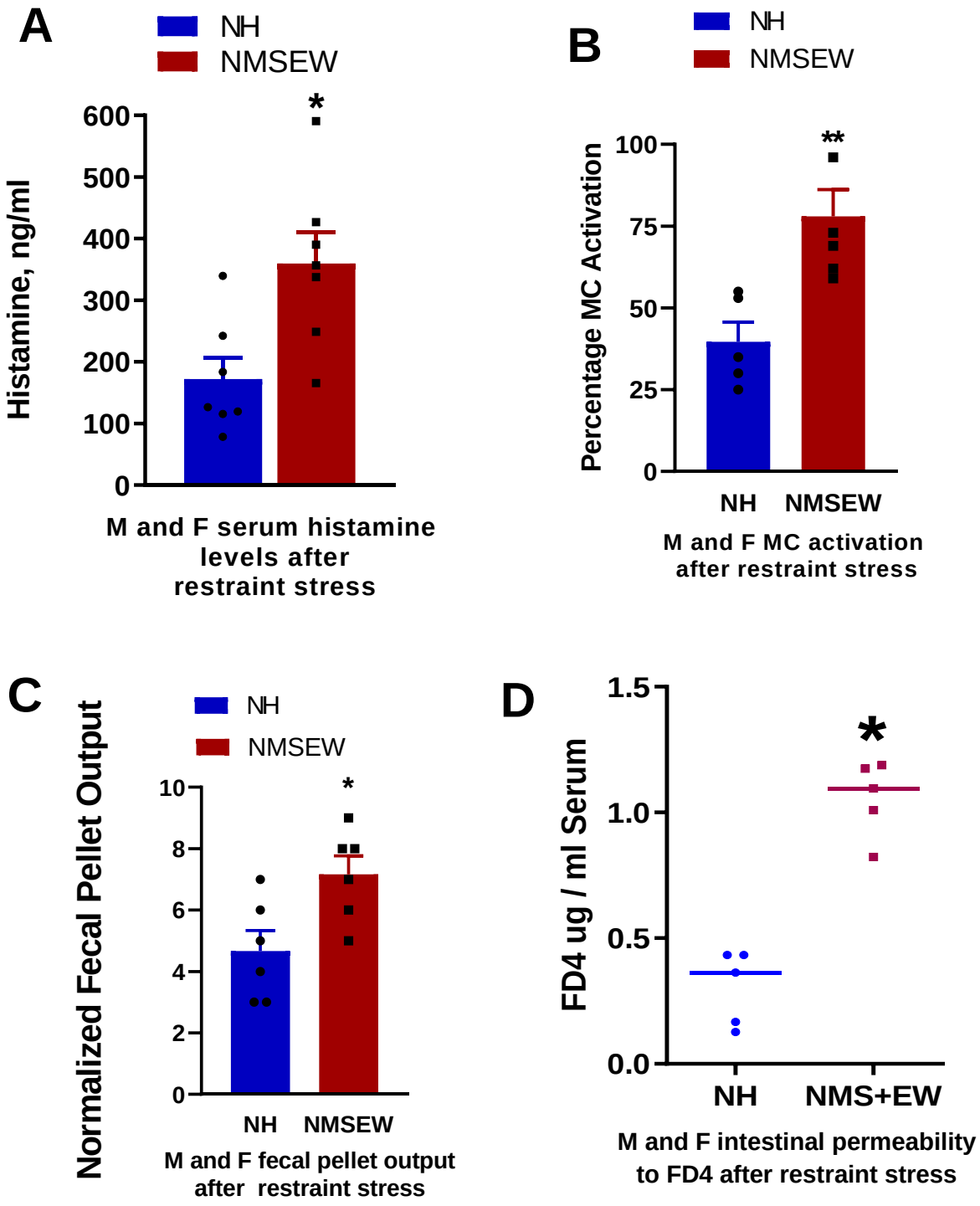
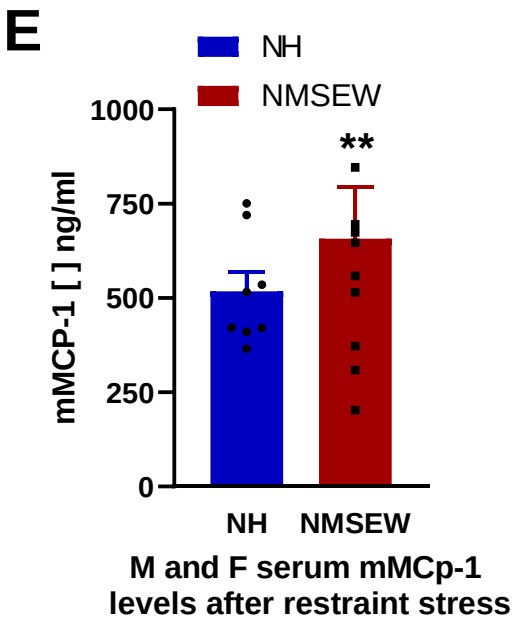


Figure 3.4 Stress-induced mast cell activation and intestinal permeability.

Figure 3.4 (cont'd)



(A) Male and female combined serum histamine levels after restraint stress. **(B)** Male and female percentage of mast cell activation in mesenteric windows from jejunum after restraint stress **(C)** GI motility **(D)** Male and females combined intestinal permeability. **(E)** Male and female Mast cell protease-1 measurement in serum after RS protocol. GraphPad Prism 8.1.0, values represent mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001.Statistical analysis used, unpaired t-test, Two- way ANOVA with Sidak's multiple comparison test. GraphPad Prism 8.1,0, values represent mean ± SEM, n = 6-10 mice per group.

Early Life Adversity Increased Severity of Symptoms in IgE-mediated Anaphylaxis

To further investigate whether NH and NMSEW exhibited differences in MC activation upon immunological stress we used a well-known MC dependent model of PSA. For

this, NH and NMSEW mice were sensitized 24 hours prior with anti-DNP IgE monoclonal antibody, via i.p. injection. Twenty four hours later they were challenged with the antigen, DNP to induce an anaphylactic response. Clinical scores and body temperature were measured as well as serum histamine and serum mast-cell protease-1 to measure MC activity. Mesenteric windows from jejunum were also collected and stained with toluidine blue to assess tissue MC activation. There was a trend for NMSEW to exhibit increased PSA-induced hypothermia at 30 min with a high dose and 60 min with a low dose post-injection with antigen. NMSEW exhibited increased clinical scores compared to controls in both doses at 15 minutes post-injection; additionally around this same time hypothermia seem to begin to differ between treatment groups. Unfortunately, two doses were used for this experiment and this decreased the power for this particular experiment. Additionally we collected blood at 30 minutes post challenge, and symptoms begun to ameliorate around that time, suggesting an earlier time point might be needed to evaluate histamine levels. There was no difference in serum histamine concentrations or mMcP-1, also no differences in MC activation on mesenteric windows. Although there is a clear sex bias with females having greater levels, no effects of treatment or sex were observed but may be due again to insufficient powering of the study and unequal number of both sexes. (**Fig. 3.5**).

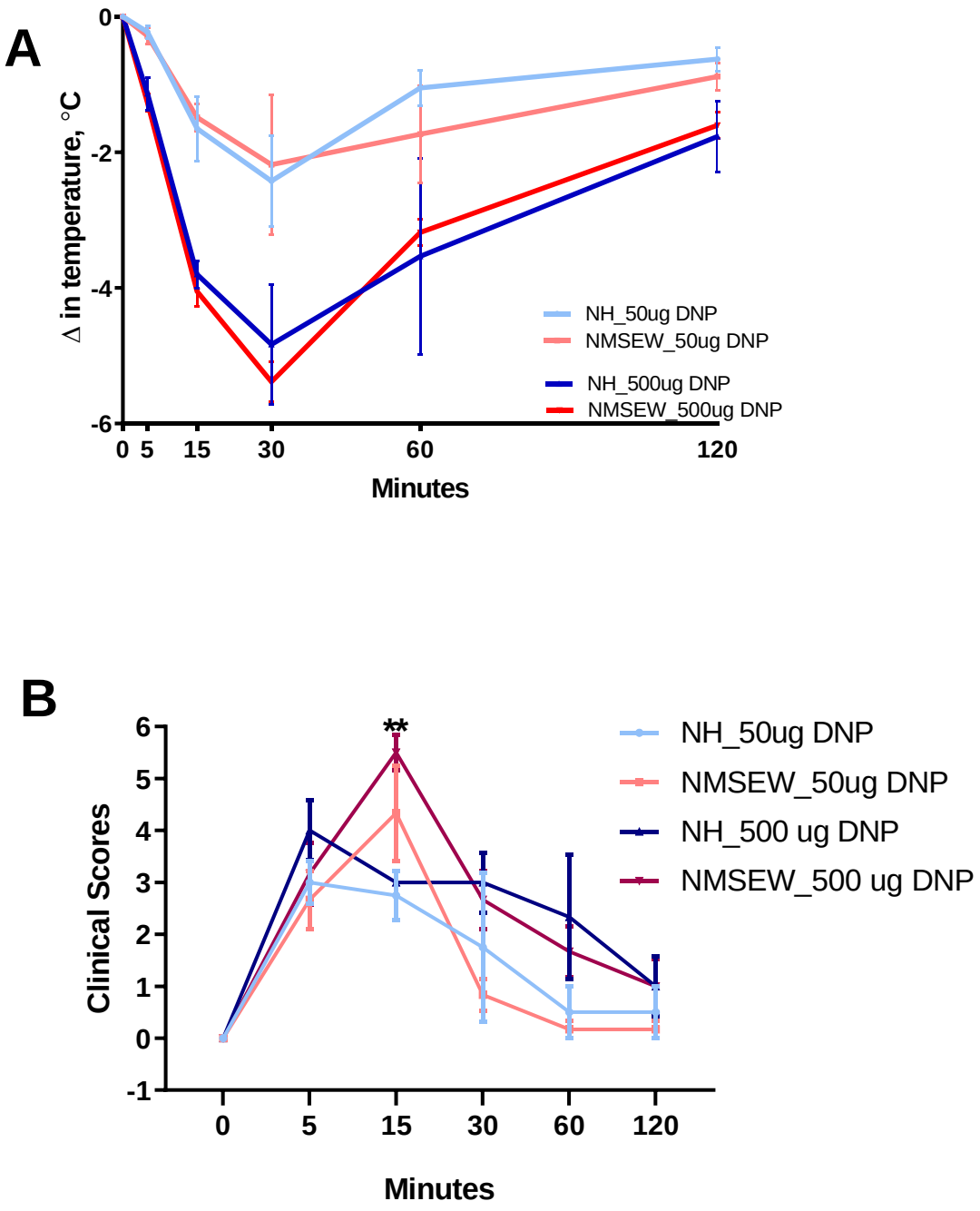


Figure 3.5 Passive systemic anaphylaxis (PSA) in NH and NMSEW mice.

Figure 3.5 (cont'd)

NH and NMSEW mice were sensitized 24 hours prior with anti-DNP IgE monoclonal antibody, via i.p. injection. Twenty four hours later they were challenged with the antigen, DNP to induce an anaphylactic response. **(A)** No differences in PSA-induced hypothermia between NH and NMSEW mice. **(B)** Increased severity of PSA-induced symptoms at 15 minutes. Two-way RM ANOVA with Tukey's multiple comparison tests.

Tissue Resident Mast Cells from Mice Exposed to ELA had Increased Preformed Mediator Content

To further evaluate mediator content differences and assess whether the heightened tissue MC activation was due to an increase in the number of MCs, we isolated tissue-resident MCs from NH's peritoneal cavity NMS+EW mice and measured their histamine content. Results are in line with the previous MC hyperactivity seen in tissue resident MCs of mesenteries and higher serum histamine. More significant histamine content was observed in NMS+EW pMC's compared to NH pMC's (**Fig 3.6**)**.**

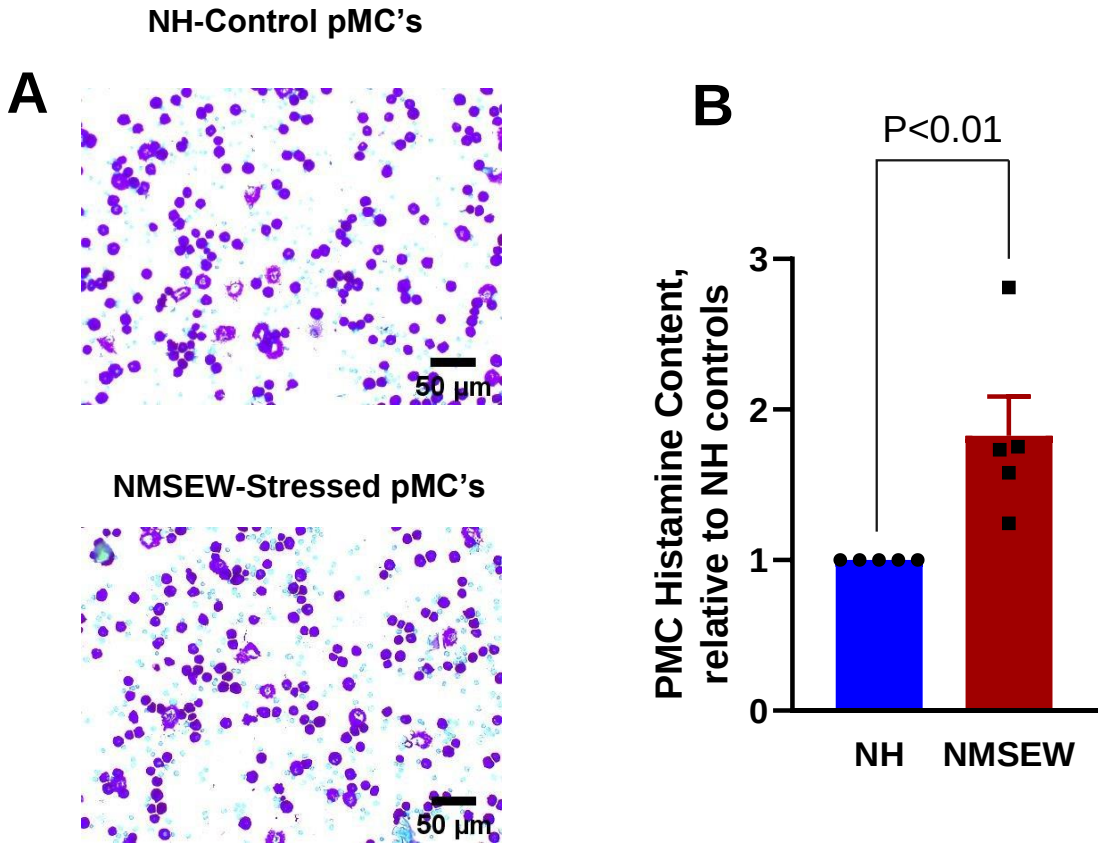


Figure 3.6 Histamine concentration in peritoneal tissue MCs from NH (Normal Handled) and NMSEW (Neonatal maternal separation +Early Weaning) mice.

Peritoneal cells were harvested from 10 week old female and male C57BL/6 mice by performing peritoneal lavage two lavages were taken from each animal and 2-3 animal lavages were pooled. Peritoneal mast cells (pMC's) were isolated using a 70% Percoll gradient. Purity of separation of pMC's was confirmed to be >95% by staining with toluidine blue. **(A)** No differences in morphology or number in PMCs from NH and NMSEW**. (B)** The total histamine content of NMSEW pMC's was higher that of NH pMC's. Unpaired Student's t-test. GraphPad Prism 8.1,0, values represent mean ±

Figure 3.6 (cont'd)

SEM. of peritoneal mast cells recovered and pooled from four-10 week old female and male C57BL/6 mice.

Discussion

Early life adverse events, such as neglect, emotional or physical abuse, have profound psychological, emotional and immunological lasting consequences in the offspring. In humans, ELA can lead to increased vulnerability to inflammatory disorders including allergies, cardiovascular, metabolic, and gastrointestinal [14-19]. Importantly, many of these diseases will go and develop in adolescence or adulthood long after the traumatic events have occurred. Particularly, there is an increasing concern for the number of child abuse and maltreatments in US alone, as this in turn, elevates the number of individuals in the vulnerable population category with impaired neuronal-immune regulation, and therefore, is a population that could largely benefit from a therapeutic intervention during a critical period. Few studies have focused on the mechanistic impact of ELA on adult immune responses and later disease susceptibility.

In the present study, we demonstrate that ELA administered during the postnatal period PD 1-17 increased basal serum histamine levels and increased MC number, histamine content and MC activation in tissue. There is growing evidence that MCs may participate in the development and pathophysiology of stress-induced disorders. However, the effects of early life stress on MC phenotype and function are yet to be elucidated. Prior NMS studies have noted the involvement of MCs as well as different pro-inflammatory cytokines NGF, IL6, IFNγ and IL1-B in the development of severe symptoms of colorectal sensitivity and gastrointestinal disorders. [20-23]. H1R receptors

on submucosal neurons play a role in the crosstalk between the ENS and MCs. [24]. Additionally, neonatal MS has shown to induce activation of MCs in the of PVN brain area with an induction of inflammatory cytokines which are responsible of communicating to nearby neurons and ultimately, directing toward an increased visceral sensitivity later in life [25]. Previous studies have also demonstrated the long-lasting effects of ELA on circulating inflammatory markers, symptoms severity and increased susceptibility to inflammatory diseases later in life such as, cardiovascular, arthritis, diabetes [1]. Moreover, modulation of the immune system by the neuroendocrine system has been previously studied; injuries to brain, specifically, limbic and hypothalamic have led to impaired immune responses. Removal of neuroendocrine organs has been shown to induce morphological changes in lymphoid organs. [26].

In the present study, we showed that NMSEW mice exhibited increased IgE-mediated anaphylaxis (clinical scores) compared to controls. Later, in our study exposure to chronic mild stress (RS) exacerbated MC responses, demonstrating increased serum histamine levels and MC activation in tissue with increased fecal pellet output and intestinal permeability compared to non-stressed mice and compared to baseline levels. This finding broadly supports the work of other studies in this area linking early life stress, and immune responses. Cohen et al., 1991 revealed that people with a history of psychological stress had increased cold viruse infection rates and respiratory lung severity. Similarly, Persinger et al., 1992 demonstrated an increase in allergy susceptibility after induction to NMS protocol. [2]. In line with these studies, maternal separation increased encephalomyelitis susceptibility and increased

neurotrophic factors of neuronal plasticity and survival of immune cells, including MCs, in adult rats. [3].

Bone marrow derived MCs can transiently be found in circulation but are not located in peripheral tissue. Therefore they cannot account for inflammatory processes within tissue. For this peritoneal MCs are ideal, pMC’s are mature serosal type of MCs the represent less than 5% of cells in the peritoneal fluid of mice. They are capable of releasing copious amounts of granules and mediators within minutes and can efficiently trigger an inflammatory response. In our study, we wanted to investigate further the ELA-induced MC hyperplasia we also demonstrated that neonatally stressed mice had increased histamine content in their pMC’s compared to non-stressed mice. We evaluated mediator content differences and assess whether increased tissue MC activation was due to an increase in the MC number or mediator content, therefore we isolated tissue-resident mast cells from NH's peritoneal cavity NMSEW mice and measured their histamine content. Histamine is a potent pre-stored MC mediator and released into the circulation very quickly and known to increase in plasma within minutes. [27, 28] Histamine is a powerful MC recruit and chemoattractant. It has been implicated to act on sensory neurons increasing sensibility to pain, vascular permeability and vasodilation. [29-32]. Once in circulation histamine is capable of binding to its receptors H1R-H4R located on various cells where it can mediate and induce inflammatory conditions such as allergies and colitis mainly through H1R and H4R activation. Furthermore, early life stress has been shown to promote MC-glial interactions mainly through H1R. In line with other studies we found that ELA induced

increase GI motility and intestinal permeability in stress mice compared to non-stressed mice.[33, 34].

Conclusion

Overall these data demonstrates that our model of NMS plus early weaning in mice is capable of inducing profound changes in MC mediator contact and responses in adulthood, long after the stress has occurred, which led us to hypothesize that ELA is inducing enduring changes in MC progenitors programming them toward a hyperactive phenotype later in life. Future studies should focus on the changes induced by ELA that are leading MC progenitors to this hyperactive phenotype. Further phenotypic and genotypic characterization of bone marrow derived MC progenitors is needed. Furthermore, deciphering the mechanism by which ELA is inducing changes in MC progenitors will help develop a targeted intervention during a critical period, or conversely, elucidating the mechanism of MC hyperactivity can help develop strategies to boost the immune responses in immunosuppressed or immunocompromised conditions.

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CHAPTER FOUR

Early life Stress Programs Mast Cell for Enhanced Degranulation, Preformed Mediator Release and De Novo Synthesized Mediator Release Upon IgE-mediated and Non-IgE-mediated Stimulation

ABSTRACT

Early life adversity (ELA) is a significant risk factor in the onset and exacerbation of inflammatory disorders, including allergy, asthma and chronic pain disorders such as irritable bowel syndrome (IBS). Mast cells (MCs) are innate immune cells involved in health and disease and have been found to be increased in number and/or activated in patients with FGIDs, in which gastrointestinal (GI) symptoms are more severe in patients with a previous history of ELA. Our previous studies using murine and porcine models showed that mast cells (MCs), a critical immune cell driving allergic inflammatory and chronic pain disorders, display a hyperactive tissue phenotype in adult animals previously exposed to ELA and display increased MC mediator content. The underlying mechanism by which ELA causes MC hyperactivity into adulthood is unknown. The aim of this study was to determine that ELA programs bone marrow-derived mast cell progenitors toward a hyperactive phenotype into adulthood by using an ELA model of neonatal maternal separation (NMS) plus early weaning. Pups from first parity dams (C57BL/6) were either raised under standard protocols (normal handling; NH) or subjected to 3 h periods of NMS daily on postnatal d 1-16 plus early weaning at 17 d of age. At 10 weeks of age, bone marrow MC progenitor cells were harvested from NH and NMSEW adult mice. They were cultured in IL3- and stem cell factor-containing media to generate a pure population of bone-marrow-derived MCs (BMMCs) determined by toluidine blue staining and double staining c-kit and FceR1. BMMC's were stimulated with IgE-DNP, IL33, and LPS to assess degranulation and cytokine release. Our results indicate that compared to NH, pre-stored histamine

content was higher (P=0.05) in bmmcs derived from NMSEW mice. Higher β-hexosaminidase and histamine release (P<0.05) was observed in the supernatant of bmmcs derived from NMSEW mice upon IgE-DNP stimulation; NMSEW bmmcs exhibited higher IL6 release into supernatant (~2 fold higher P<0.001) upon IL33 stimulation and higher TNF (P<0.05) and IL6 (P<0.01) release into supernatant upon LPS stimulation and this response seems to be exacerbated in NMSEW females (P<0.05). Early life stress-induced a higher IL-3 induced proliferative effect in NMSEW bmmcs. Additionally, transcriptome analysis revealed that ELA had a long-term effect in MC gene expression and that females particularly seem to be more susceptible to the induced transcriptional changes of ELA.

Taken together, these new data suggest there are ELA induced factors promoting long-lasting functional changes in MC progenitors. Future studies will investigate the mechanisms by which ELA drives MC progenitors' lasting hyperactivity and its role in MC-related disorders in adulthood. Future studies will investigate the mechanisms by which ELA drives lasting hyperactivity of bone marrow MC progenitors and tissue mast cells, its contribution to disease into adulthood, and its role in increasing the risk for MC-related disorders in adulthood.

Introduction

Exposure to ELA in the form of abuse, neglect, violence, caregiver with mental illness, death in the family, can have profound long-term effects in the stress responsive system, leading to a variety of stress related disorders such as, anxiety and depression [1], chronic GI disorders such as IBS and IBD [2-4], allergies [2, 5]. Furthermore, compelling evidence has demonstrated the link between ELA and inflammatory disorders later in life, people who suffered severe childhood trauma are more at risk for cancer, diabetes, and cardiovascular diseases [6]. Studies have demonstrated that early life stressors are likely to trigger the release of pro-inflammatory cytokines and elicit an inflammatory response. The persistence of cortisol levels causes immune cells to become insensitive to glucocorticoids, thus enhancing inflammation.

MCs are key players in the stress-induced pathophysiology, MCs have found to be activated by acute stress [7, 8]. Moreover, previous studies in our lab have demonstrated MC hyperactivity in animals subjected to early life stressors or mild adult stressors.[9-11]. It is clear and there is enough evidence to support the impact ELA has on adult inflammation, and that MCs are playing a role in stress-induced diseases. However, few studies have focused on the mechanism by which ELA is affecting MC hyperactivity in adulthood. We previously demonstrated in Chapter 3 that ELA affected MC hyperactivity and Mc mediator content in adulthood; MCs were capable of storing and pre-synthesizing more mediators as well as increasing MC activity upon subjection to adult chronic stress and immunological stress.

Rationale: Our previous studies have demonstrated that mice exposed to ELA exhibited increased adult tissue activation and pre-stored mediator content. We therefore, developed the working hypothesis that ELA is inducing phenotypic and genotypic changes in bone marrow MC progenitors programming them toward a hyperactive phenotype in later life. By utilizing our ELA model of neonatal maternal separation plus early weaning (NMSEW) in C56BL/6 mice we collected and harvested MC progenitors from ELA mice and controls that we later used to generate a pure population of MCs and later performed *in-vitro* assays as well as transcriptome analysis to investigate the genotypic changes induced by ELA. Here, we demonstrate that BMMCs from mice exposed to ELA exhibit increased release of MC mediators and inflammatory cytokines upon stimulation compared to controls. Moreover, that ELA had a long-term effect in MC and immune-relevant gene expression and that female particularly seem to be more susceptible to the induced transcriptional changes of ELA.

Methods

Isolation and generation of Bone marrow derived mast cells (BMMCs)

Bone marrow progenitor cells were harvested from femurs of 10 week old female and male C57BL/6 mice and cultured in 182-cm^2 tissue vented culture flasks with complete media (cRPMI) containing RPMI 1640 with L-glutamine (Corning® Corning, NY), supplemented with 10% heat inactivated fetal bovine serum (FBS) (GIBCO^{TM,} Waltham, Massachusetts', 100U/ml penicillin, 100ug/ml streptomycin, 1 mM HEPES, 1 mM non-essential amino acids and 1 mM sodium pyruvate (Corning® Corning, NY), 5ng/ml recombinant mouse IL-3, and 5ng/ml recombinant mouse stem cell factor (SCF) (R&D Systems® Minneapolis, MN). Cells were incubated in a humidified 5% CO2 and 95% air at 37°C. The media was changed twice a week to eliminate adherent cells by transferring the cell suspension to a 50-mL conical polypropylene centrifuge tube, and centrifuging for 5 minutes at 1,500 rpm, at 20°C. The culture flasks were changed every time the medium was changed. After 4 weeks of culture, <95% of the cells were identified as mast cell as determined by toluidine blue staining (0.5% pH 0.5) and cell-high-affinity IgE receptor surface expression of FcεRI (Phycoerythrin, PE) and c-Kit (Pacific Blue, PB) (Biolegend, San Diego, CA) was confirmed by flow cytometry (BF LSR II). Experiments were carried out within 6- 10 weeks of culture initiation. Percentages and geometric mean florescence intensity of Double positive cell population (FcεRIa+, c-kit+) were average among BMMcs from same group and later compared between groups. Experimental units of cultured BMMCS were defined by bone marrow of individual animals.

Flow Cytometry of BMMCS

FcεR1/c-kit double-positive staining by flow cytometry. The n for bone marrow-derived MC (BMMC) experiments was defined by independent flasks.

B-hexosaminidase Release

BMMCs (2.00 × 10 cells/ml) were sensitized with 1 µg/ml mouse monoclonal anti-2,4-dinitrophenol (DNP) IgE antibody ( ) and later stimulated with 0, 15.5, 31, and 62 ng/mL DNP-HSA for 1 h. The presence of β-hexosaminidase in the supernatant and cell lysate was calculated using the substrate p-nitrophenyl N-acetyl-α-D-glucosaminide. The percentage of β-hexosaminidase release was calculated as a percentage of total β-hexosaminidase content.

Histamine Release measurement

BMMCs (1 x10^6 cells/ml) were sensitized with 1 µg/ml of mouse monoclonal anti-2,4-dinitrophenol (DNP) IgE antibody (Sigma-Aldrich, St. Louis, MO,) and later stimulated with 0, 15.5, 31, and 62 ng/ml of DNP-HSA for 1 hour. Histamine levels in the culture supernatants and cell lysates (RIPA buffer lysis) were determined by (Oxford Biomedical Research, Rochester Hills, MI) competitive ELISA and mMcp-1(Biocompare, Seattle, Washington) according to the manufacturer's instructions.

IL33 stimulation

BMMCs (1 x10^6 cells/ml) were stimulated at 37°C with 10ng/ml IL-33 for 8 hours. Cell supernatants were collected and stored at -80°C. Viability of the cells throughout experiments >98% were assessed by trypan blue dye exclusion test in an automated Cell Counter (LUNA™, San Mateo, CA). IL6 cytokine was measured using IL6 ELISA (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's protocol.

LPS Stimulation

BMMCs (1 x10^6 cells/ml) were stimulated at 37°C with 500 ng/ml of LPS for 8 hours and IL6 and TNFα was quantified using ELISA and IL6 and TNFα ELISA.

Proliferation Assay

Mast cell proliferation was tested by BrdU incorporation. Briefly, cells were grown at a density of 2 x 10^5 cells/mL in 100 µL/well of cRPMI in a 96-well plate according to manufacturer's instruction. After 24 h of treatment, cell proliferation was measured using the BrdU Cell Proliferation ELISA (abcam) according to the manufacturer's protocol. The absorbance of each well was determined at a wavelength of 450/550 nm using an automated microplate reader. The second wavelength was subtracted from the first; effectively subtracting out the background noise that is usually not wavelength specific. Wells with no cells (media alone), well with cells but no BrdU (assay background) and well with no IL3 (growth arrest) were set aside as negative controls.

In Vivo-LPS Challenge

10 wk old NH and NMSEW male mice were i.p injected with LPS (1mg/ kg; Sigma) or saline. Rectal temperatures and clinical scores were scored every hour for 6 hours.

Capillary western blot (Wes) analysis of TLR4

Capillary Western analysis was performed using the Protein Simple Wes System following manufacturer's instruction. Briefly, samples (BMMCs extracts) were diluted with 0.1 x sample Buffer. Later, 4 parts of diluted sample were combined with 1 part of fluorescent Master Mix (sample buffer x fluorescent standard x 200mM DTT). After denaturation, samples were blocked and primary antibody 1:50 for Rabbit Anti-TLR4

antibody (ab13556, Abcam, Cambridge, UK) , HRP conjugated secondary antibody and chemiluminescent substrate were dispensed in designated wells. A biotynated ladder provided the molecular weights. Recombinant human TLR4 protein (Active) (ab233665) was used as control.

RNA Isolation, High-throughput Sequencing, and Transcript Analysis

RNA was isolated from unstimulated BMMCs ($1x10^7$ cells/ml) using the RNeasy minikit (QIAGEN) adding the DNase digestion step according to the manufacturer's instructions. RNA quality and concentration was verified using the NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) 260/ 280 ratio was used to check quality. Total RNA was sent to the Van Andel Institute for library preparation and sequencing. The samples' quality was verified using a bioanalyzer; only samples with acceptable RIN values were further processed. Raw data were quality controlled with FastQC v0.11.5. The R1 has high quality for all samples. Adapters were removed with Trimgalore v0.4.4_dev (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and mapped with STAR v2.5.2b [12] to the mm10 genome. STAR outputs counts for all genomic features/tags/genes (option: –quantMode GeneCounts). These are the raw data imported into this R pipeline, which uses the edgeR [13] framework for RNAseq analysis. First, however, genes with low counts and unlikely to be translated (biologically meaningful) and also without enough counts for a reliable statistical judgment are removed. We require genes to have greater than (10/minimum sample library size in millions) counts per million in two samples. The DGE analysis was performed using the edgeR framework. The TMM normalization for library size and composition bias was applied to the count-filtered data. This was followed by the

models and tested using the quasi-likelihood F test. P-values were then adjusted using the BH method. Genes were termed significant if they passed the FDR and logFC thresholds. Significant genes were used in the gene set enrichment analysis using the R package goseq, which adjusts for gene length. Both the Reactome Pathway and Gene Ontologies were tested for enrichment.

Results

Early life Stress Programs Bone-Marrow derived Mast Cells for Enhanced Degranulation, and Preformed Mediator Release

For the following experiments, we utilized 10-week old bone marrow-derived mast cells (BMMCS) from NH and NMS+EW mice. After verifying purity with flow cytometry and toluidine blue staining. We first evaluated if there were differences in the expression of c-*kit* and FCeRI. Our results show there were no differences in receptor expression between NH and NMSEW BMMCs. No morphological differences were observed between NH and NMS+EW BMMCs upon visualization with toluidine blue. **(Figure 4 .1)**

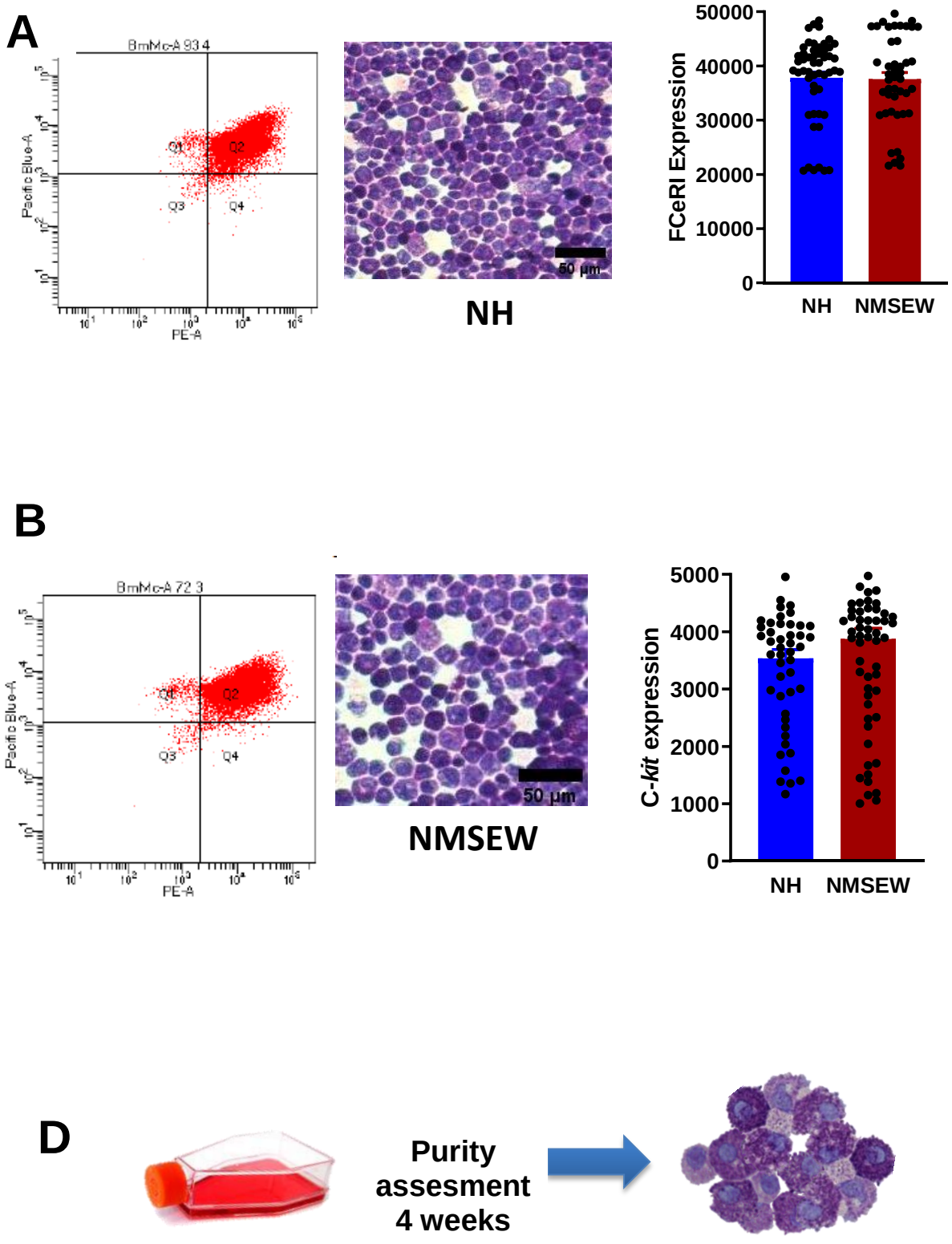


Figure 4 .1 Mast cell Receptor expression between NH and NMSEW BMMCs.

Figure 4.1 (cont'd)

BMMCs were derived from femurs of 10 wk old NH and NMS+EW male and female C57BL/6 mice, progenitors cells were cultured for 4 weeks and stained with fluorescent-conjugated c-kit (Pacific Blue) and FcεRIα (PE) antibodies.

(A) Representative images of flow cytometry, the horizontal axis represents FcεRIα, and the vertical axis represents c-kit receptor expression. Double positive (c-kit+ FcεRIα+) cell percentages were not different between BMMCs derived from NH and NMS+EW. **(B)** Comparison of c-kit geometric mean fluorescence intensity (MFI) indicates no significant differences between NH and NMS+EW BMMCs. Unpaired Student's t-test. **(C)** Comparison of FcεRIα geometric mean fluorescence intensity (MFI) shows no significant differences between NH and NMS+EW BMMCs. Unpaired Student's t-test. **(D) Schematical Representation** of BMMC purity assessment at 4 wks of culture stained with toluidine blue at 4 weeks. There were no morphological changes between NH and NMS+EW BMMCs. GraphPad Prism 8.1,0, values represent mean ± SEM. of a 4 independent experiments with 6 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

To evaluate if there were differences in the proliferative capacity of BMMCs, proliferation was assessed by BrdU incorporation. Our results indicate that early life stress induced a higher IL-3 induced proliferative effect in the bmmcs. (**Figure 4.2)**

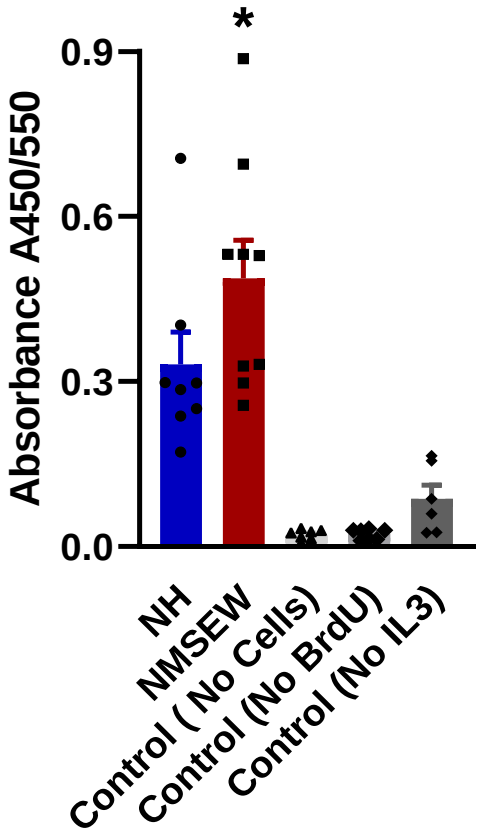


Figure 4.2 IL3- Induced proliferation of NH and NMSEW BMMCs.

NH and NMSEW BMMCs proliferation was tested by BrdU incorporation, At a density of 2 x 10^5 cells/mL in 100 µL/well of cRPMI in a 96-well. After 24 h of treatment, cell proliferation was measured using the BrdU Cell Proliferation ELISA. The absorbance of each well was determined at a wavelength of 450/550 nm using an automated microplate reader. The second wavelength was subtracted from the first; effectively subtracting out the background noise that is usually not wavelength specific. Wells with no cells (media alone), well with cells but no BrdU (assay background) and well with no

Figure 4.2 (cont'd)

IL3 (growth arrest) were set aside as negative controls. GraphPad Prism 8.1,0, values represent mean ± SEM. of a representative experiment with 6 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

We then evaluated histamine content and histamine release upon IgE stimulation in BMMCs. For this, bmmcs were sensitized overnight with DNP-specific IgE (1ug/ml); the next day after, the antigen (DNP-HSA) was administered to the cell culture to induce FceR1 cross-linkage and degranulation. We then evaluated MC β-hexosaminidase (a well-known granule marker of degranulation) and histamine release (MC mediator, an MC activity indicator) in the supernatant. Compared to NH, pre-stored histamine content was higher (P=0.05) in bmmcs derived from NMS+EW mice. Higher β-hexosaminidase and histamine release (P<0.05) was observed in the supernatant in bmmcs derived from NMS+EW mice upon IgE-DNP stimulation; no significant differences were observed in β-hexosaminidase release in unstimulated conditions. **(Figure 4.3)**

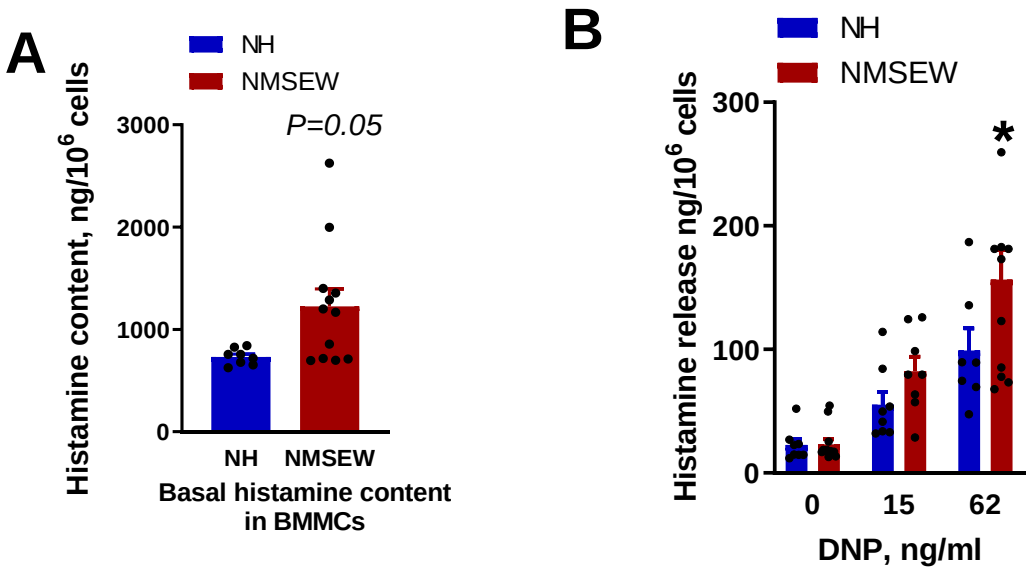
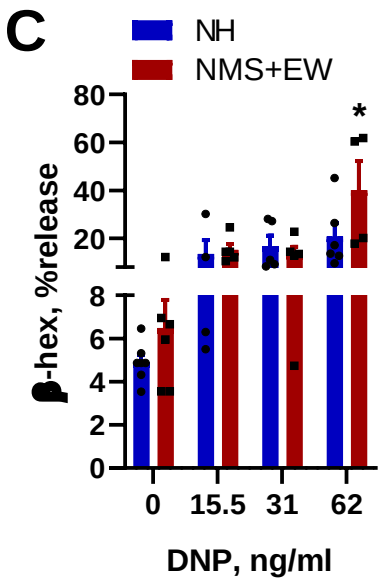


Figure 4.3 Histamine content and histamine release upon IgE stimulation in NH and NMSEW BMMCs.

Figure 4.3 (cont'd)



BMMCs were sensitized with 1 µg/ml of mouse monoclonal anti-2,4-dinitrophenol (DNP) IgE antibody and later stimulated with 0, 15.5, 62 ng/ml of DNP-HSA for 1 hour. Histamine levels in the culture supernatants and cell lysates were later determined. **(A)** Total histamine content was greater in BMMCs derived from NMSEW mice compared to BMMCs derived from NH mice. Unpaired Student's t-test. **(B)** BMMCs derived from NMSEW mice released greater histamine amounts after FcεRI stimulation and cross-linkage with antigen than BMMCs derived from NH mice. Two-way ANOVA with Sidak's multiple comparisons test. **(C)** BMMCs derived from NMS+EW mice had increased release of β-hexosaminidase after DNP-HSA stimulus (62 ng/mL). Two-way ANOVA with Sidak's multiple comparisons test. GraphPad Prism 8.1,0, values represent mean ± SEM. of a representative experiment with 6 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

Additionally, we evaluated mMCP-1 (mast cell protease-1) and TNFa release upon IgE stimulation. BMMCs derived from NMSEW mice exhibited higher basal TNFα release and mast cell protease-1 release into supernatant (P<0.05) upon IgE-DNP stimulation. **(Figure 4.4)**

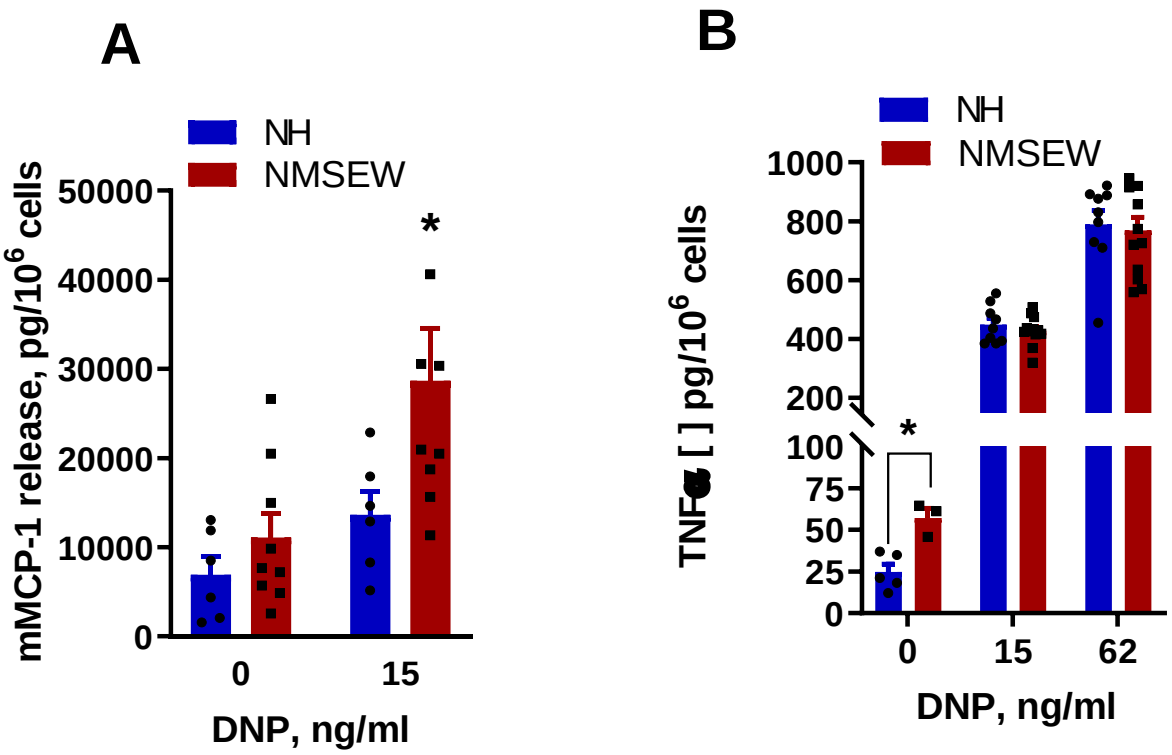


Figure 4.4 Mast cell protease-1 and TNFa release upon IgE stimulation in NH and NMSEW BMMCs.

BMMCs were sensitized with 1 µg/ml of mouse monoclonal anti-2,4-dinitrophenol (DNP) IgE antibody and later stimulated with 62 ng/ml of DNP-HSA for 1 hour. **(A)** BMMCs derived from NMSEW mice exhibited greater mast cell protease-1 release into supernatant upon IgE-DNP stimulation. Two-way ANOVA with Sidak's multiple comparisons test. **(B)** BMMCs derived from NMSEW mice exhibited higher basal TNFα release into supernatant upon IgE-DNP Stimulation. Two-way ANOVA with Sidak's

Figure 4.4 (cont'd)

multiple comparisons test. GraphPad Prism 8.1,0, values represent mean ± SEM. of a representative experiment with 6 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

Early life Stress Programs Mast Cell for Enhanced Preformed Mediator and de novo Synthesized Mediator Release upon Non-IgE-mediated Stimulation

Furthermore we wanted to try other non-IgE stimulation to evaluate if the heightened response observed was specific to IgE-DNP stimulation. We stimulated BMMC with IL33, a potent inducer of MC Cytokine release and lipopolysaccharide (LPS). IL33 exerts its activity by binding to the primary receptor ST2 in mast cells triggering downstream signaling pathways. LPS interacts with toll-like receptor 4 (TLR 4) in mast cells, resulting in augmentation of MC degranulation upon antigen stimulation. BMMCs derived from NMSEW mice exhibited higher IL6 release into supernatant (~2 fold higher $P<0.001$) upon IL33 stimulation and higher TNF ($P<0.05$) and IL6 ($P<0.01$) release into supernatant upon LPS stimulation and this response seems to be exacerbated in NMS EW females ($P<0.05$). **(Figure 4.5)**

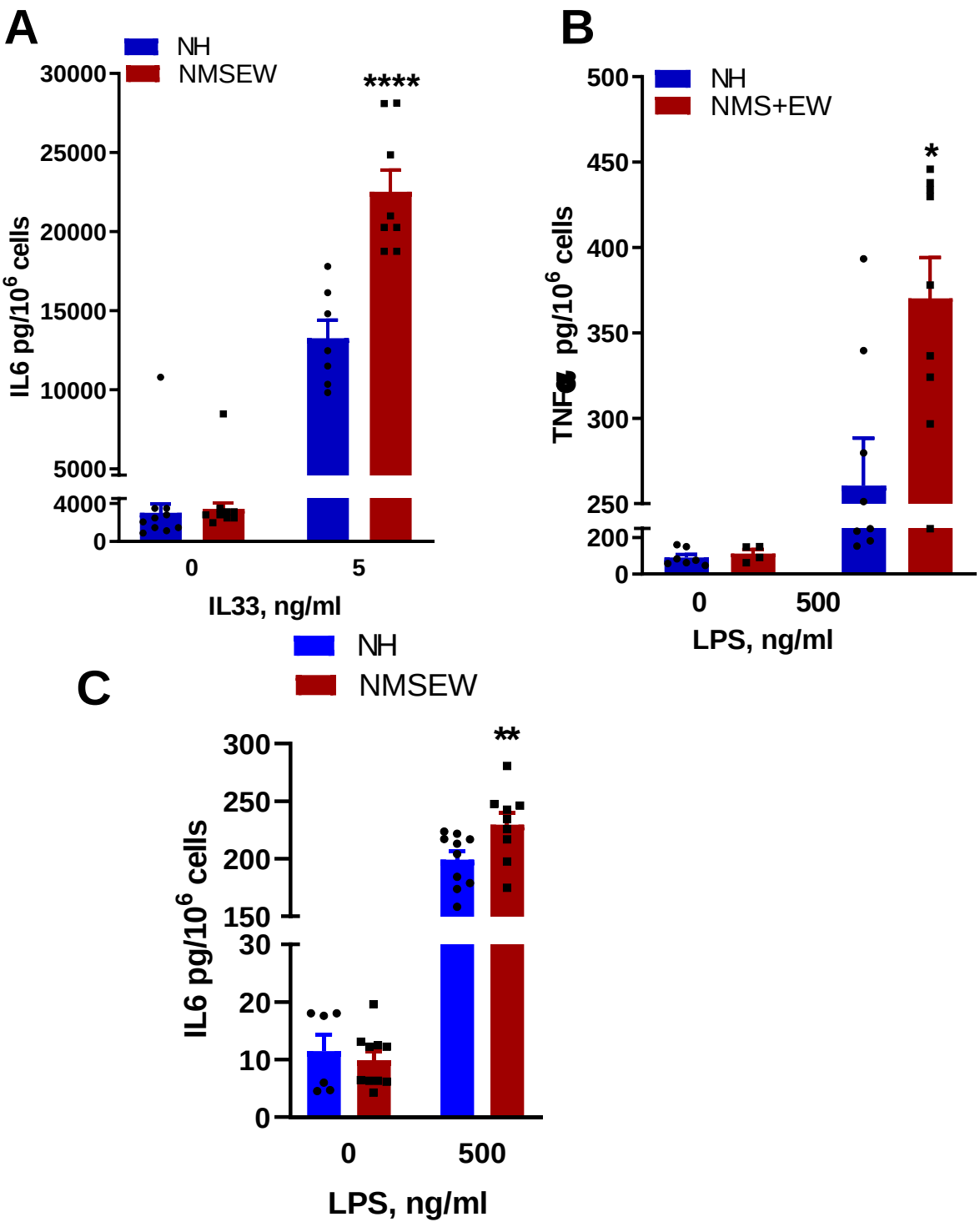


Figure 4.5 TNFa and IL6 release upon IL33 and LPS stimulation of NH and NMSEW BMMCs.

Figure 4.5 (cont'd)

BMMCs were stimulated at 37°C with 10ng/ml IL-33 for 8 hours. **(A)** BMMCs derived from NMSEW mice exhibited higher IL6 release into supernatant compared to BMMCs derived from NH mice. Two-way ANOVA with Sidak's multiple comparisons test. BMMCs were stimulated at 37°C with 500 ng/ml of LPS for 8 hours. **(B)** BMMCs derived from NMSEW mice exhibited higher IL6 release into supernatant compared to BMMCs derived from NH mice. Two-way ANOVA with Sidak's multiple comparisons test. **(C)** BMMCs derived from NMSEW mice exhibited higher IL6 release into supernatant upon LPS stimulation. Two-way ANOVA with Sidak's multiple comparisons test.

GraphPad Prism 8.1,0, values represent mean ± SEM. of a representative experiment with 4 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

ELA Induces Transcriptional Changes in Bone Marrow-Derived Mast Cell Progenitors

To gain more understanding of the basal gene expression changes induced by ELA on adult MCs, we profiled the gene expression patterns in MCs from NH and NMSEW mice by RNA sequencing. Briefly, MC progenitors were extracted from the femurs of NH and NMSEW to generate a pure population of MCs. After confirming MC purity ~6wks, RNA was extracted from unstimulated BMMCs (1.0×10^7 cells). After verification of RNA quality, extracted RNA samples were sent to the Van Andel Institute for transcriptome analysis and further bioinformatic analysis. Notably, a total of 2,093 genes were differentially expressed in NMSEW compared to controls. RNA sequencing revealed that 1,203 genes were altered by ELA in males and 890 genes in females **(Fig 4.6)**

Principal component analysis identified that transcripts from NMSEW grouped differently from controls, and that within the NMSEW group, females grouped

independently **(Figure 4.7)**. Suggesting, that ELA had a long-term effect in MC gene expression and that females particularly seem to be more susceptible to the induced transcriptional changes of ELA. Remarkably, we found upregulation in *Lipn* (2.70 fold); this gene encodes for lipases. It is involved in lipid metabolism [14]; similarly, we found that *CD36* gene expression was 3.36 fold higher in NMSEW BMMCs; CD36 is a gene that encodes for a fatty acid transporter and plays a role in obesity and diabetes [15, 16], suggesting a role of ELA in lipid mobilization and probably predisposition to metabolic-related diseases.

These finding goes in line with our finding of increased body weight in NMSEW mice particularly females (**Fig.S.1.C**). Also, upregulated was *vasn* gene expression (2.20 fold) in NMSEW BMMCs compared to controls; vasorin (*vasn*) has been found to facilitate interactions between the nervous system and vascular system [17]. Interestingly, we found that *TLR4*, *TLR7*, *and TLR8* gene expression to be 2-folds higher in NMSEW BMMCs; similarly, the TNF receptor family was also upregulated NMSEW BMMCs. Toll-like receptors in MCs play an important role in pathogen defense, but also have found to synergistically interact to exacerbate IgE-mediated responses and enhanced pro-inflammatory cytokine production[18]. The TNF receptor superfamily activates NFKB, an essential receptor in innate immunity, and the TNF receptor family also plays a role in autoimmune disorders. [19] Remarkably, the expression of both toll-like receptor family and TNF family was found to be higher in NMSEW female BMMCS. (**Fig 4.5**)

Additionally protein expression of TLR 4 was found to be increased in bone-marrow MCs from early life-stressed mice. Moreover, MCs from stressed animals also

show differences in proteases and metalloproteinases; such as Mcpt4, *Mcpt8*, and *Mcpt2, Mmp13, Mmmp19,* which are involved in tissue remodeling, repair, and inflammation [20-22]. An increased gene expression of Interferon regulatory factor 6 (*Irf6*) was observed in NMSEW BMMCS. This particular gene is very important in early development it encodes a transcription factor that regulates the production of a set of inflammatory cytokines responsible for alleviating viral and bacterial infections, found to play an important role in LPS responses [23] and also, recently found to be involved in the pathogenesis of systemic sclerosis [24]. Other abundantly expressed genes in NMSEW BMMCs were *Mab21l2* (3.6 fold) and *Rgs13* (2.6 fold) involved in regulating B-cell responses to cytokines and G-couple receptor-mediated responses in MCs respectively. Additionally, Mab21l2 has been found to play a role in cell fate and cell proliferation [25-27] (**Fig 4.17**). Of particular interest was the canonical pathway analysis which demonstrated an increase in genes involved in NFKB signaling, integrin signaling, and migration of leucocytes.

Overall, these findings indicate that MCs derived from ELA-stressed mice have different gene expression patterns with an increased upregulation in immune-MC-related genes and lipid metabolism genes and that female seem to be more susceptible to ELA induced gene expression changes.

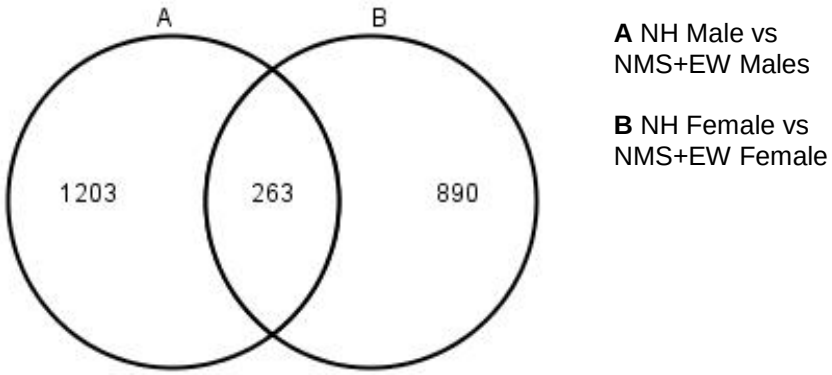


Figure 4.6 Venn diagram of commonly expressed genes in females and males NMSEW BMMCs compared to NH.

Venn diagram illustrating differential gene expression NMS females compared to NH females and NMS males compared to NH males. A is the number of differentiated genes in Males, B is the number of differentiated genes in female.

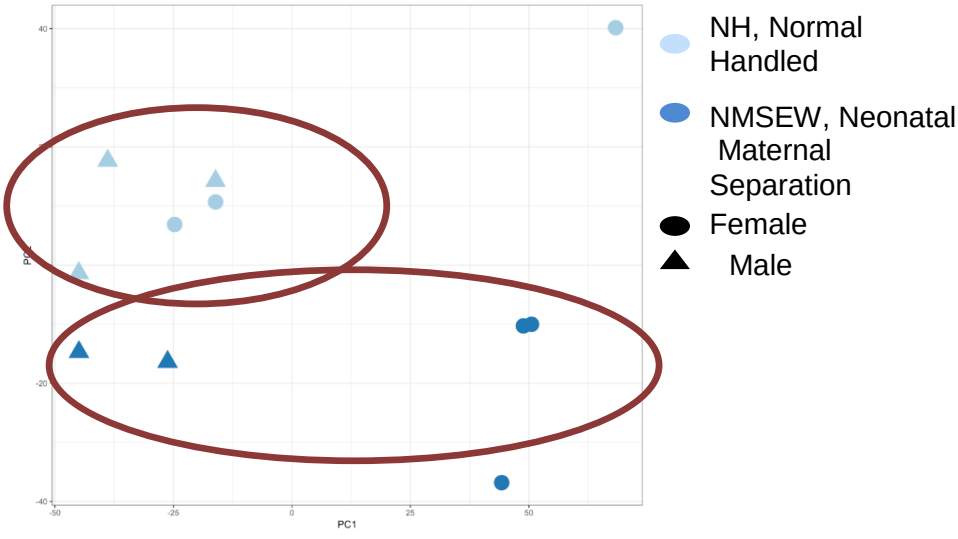


Figure 4.7 Principal component analysis (PCA) of unstimulated BMMCs from NMSEW and NH mice.

Figure 4.7 (cont'd)

PCA of the normalized RNA Seq data transcripts, raw data were quality controlled with Fastq. The R1 has high quality for all samples. Adapters were removed with Trimgalore and mapped with STAR. Light blue represents NH BMMCs, dark blue represents NMSEW BMMCs. Circle represents females, and triangle represents males.

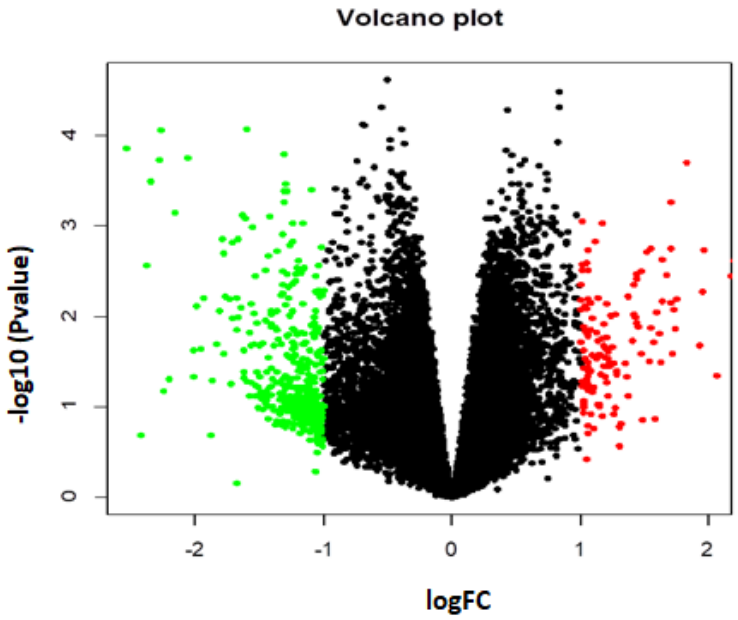


Figure 4.8 Volcano plot of differentially expressed genes of BMMCs from NMSEW compared to NH

Figure 4.9 Comparison of Biological Function Revealed by RNA-Seq analysis between NMSEW BMMCs compared to NH.

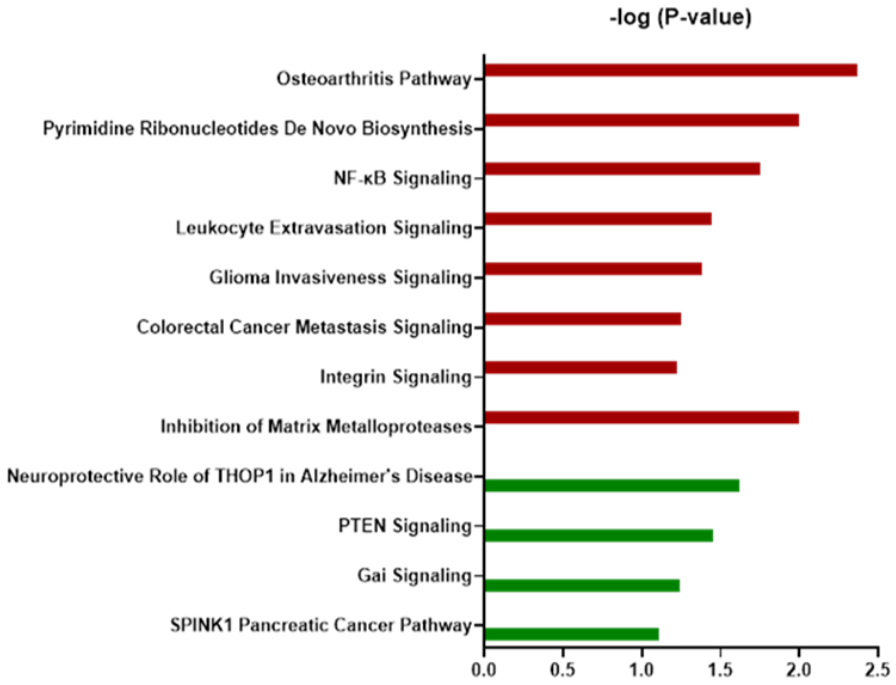


Figure 4.10 Significant Canonical Pathways in NMS compared to NH based on z-score ranked based on –log (p-value).

Figure 4.10 (cont'd)

Ingenuity pathway analysis showing the most highly scoring canonical pathways.

Canonical pathway analysis on the gene transcripts using ingenuity pathway analysis (IPA) revealed most significant enrichment in pathways related to pyrimidine rubonucelotide de novo byosynthesis, NFKB singnaling (including TNf receptors and TLRS, Integrin Signaling, differences in leukocyte signaling. differences in metalloproteases.

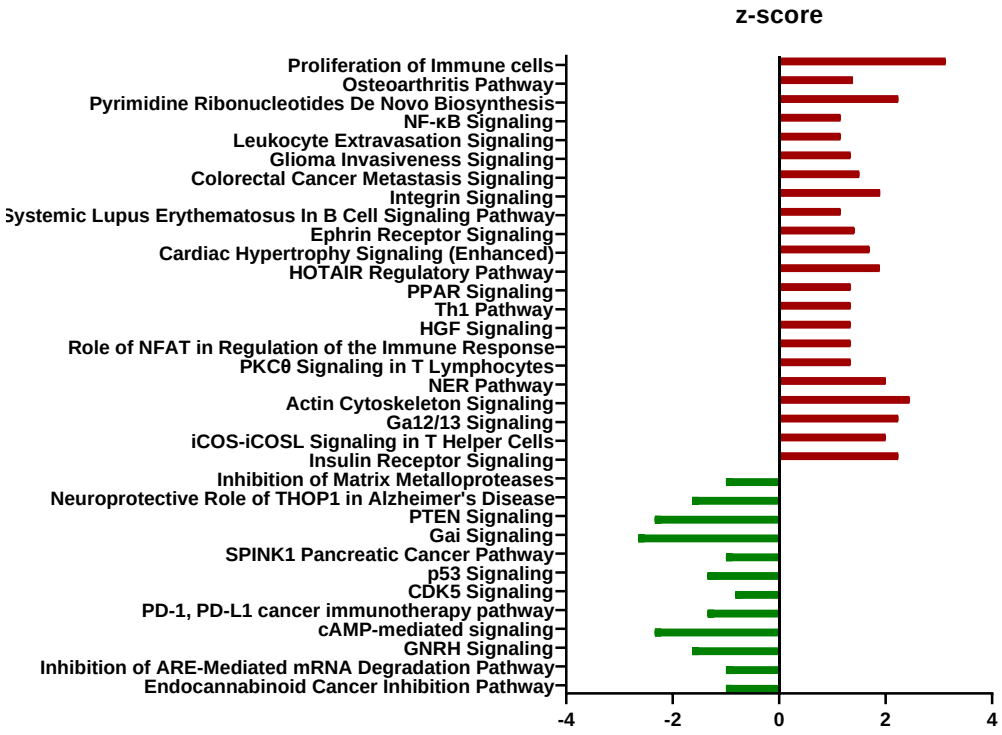


Figure 4.11 Canonical Pathways in NMS compared to NH based on Z-score.

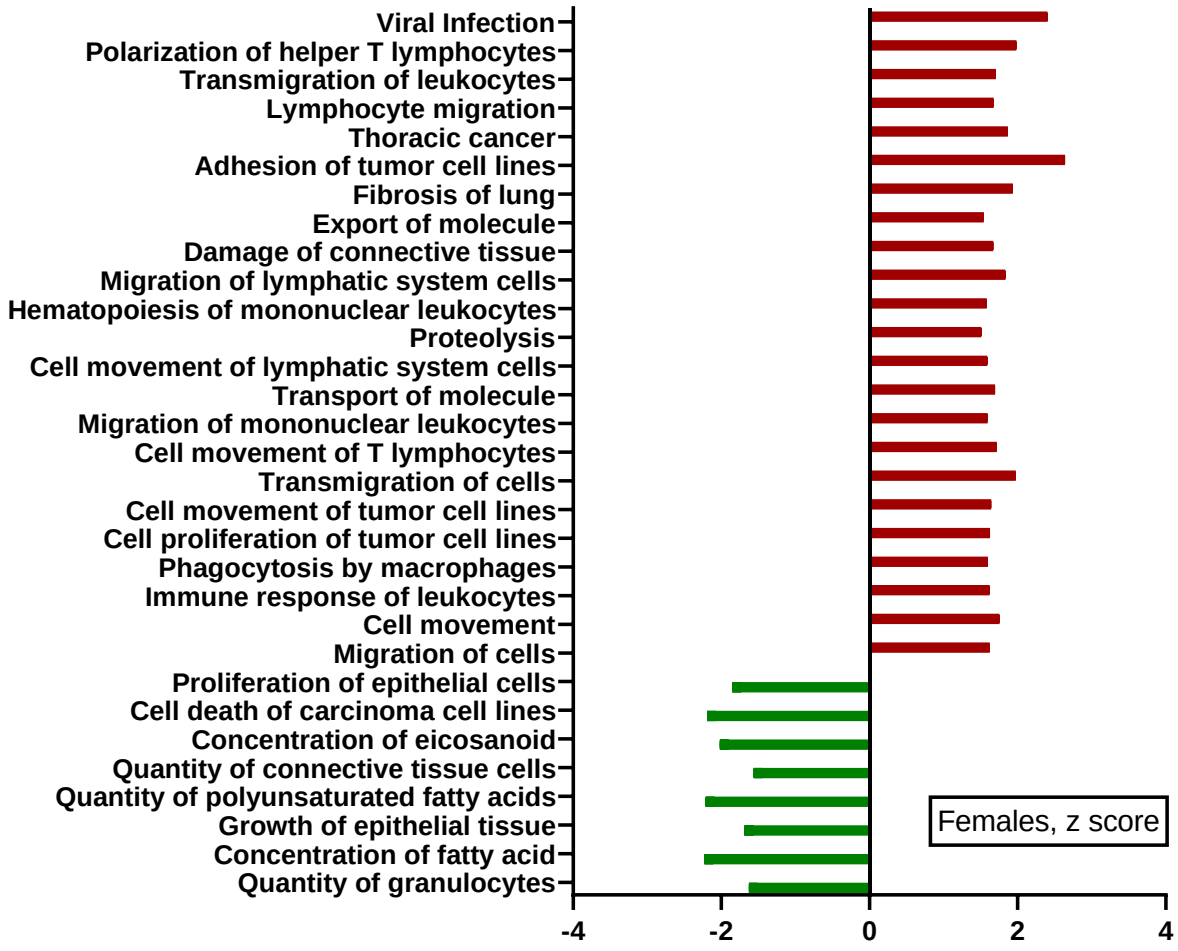


Figure 4.12 Significant Biological Processes in Diseases and Functional categories in NMS Females compared to NH Females.

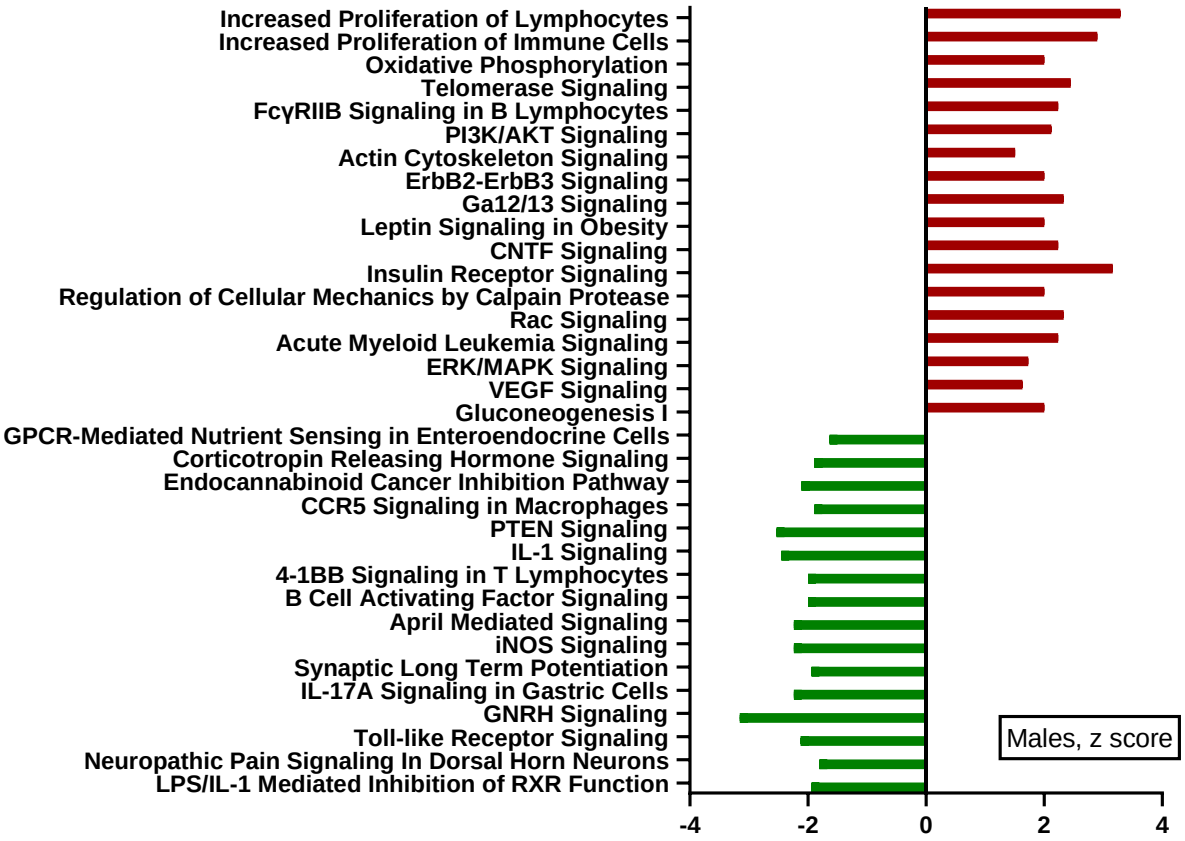


Figure 4.13 Significant Biological Processes in Diseases and Functional categories in NMS mice Males compared to NH males.

Female BMMCs

Top Upstream Regulators	P-value
Females	
IL13	3.26 E-12
GATA2	4.33E-12
TCL1A	6.20E-11
IL4	2.56E-10
Lipopolysaccharide	7.87E-10

Male BMMCs

Top Upstream Regulators	P-value
Males	
TP53	2.39E-12
APP	9.24E-10
ERBB2	1.41E-09
progesterone	1.95-0.9
Beta-estradiol	2.27E-0.9

Figure 4.14 Top Upstream Regulators in Female and Male BMMCs

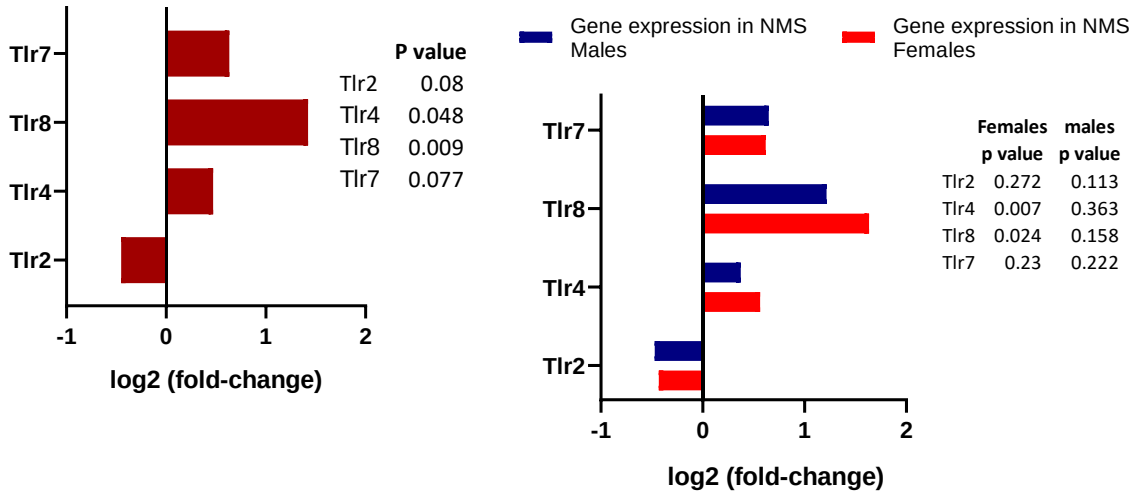


Figure 4.15 Gene expression of Toll-like receptors in NMSEW BMMCs Relative to NH BMMCs.

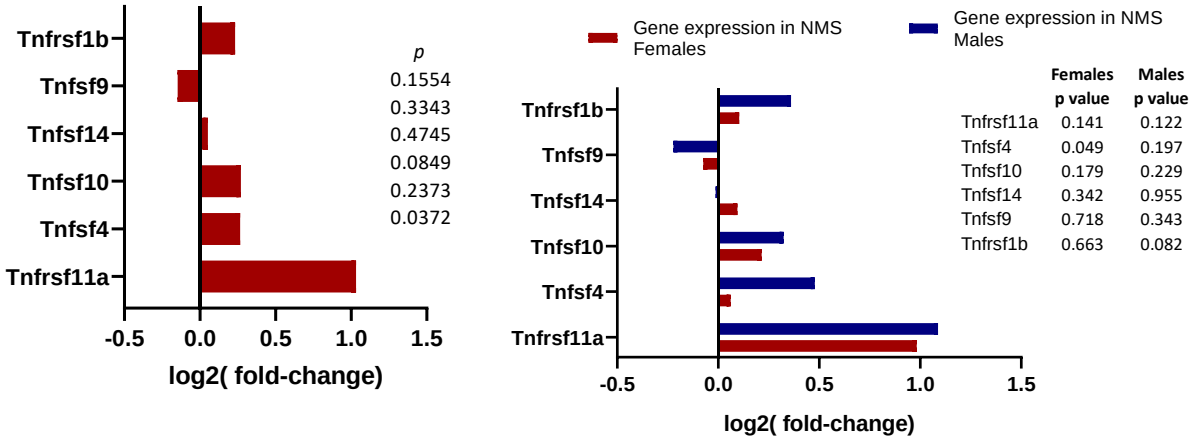


Figure 4.16 Gene expression of TNF Family receptors relevant genes in NMSEW BMMCs Relative to NH BMMCs.

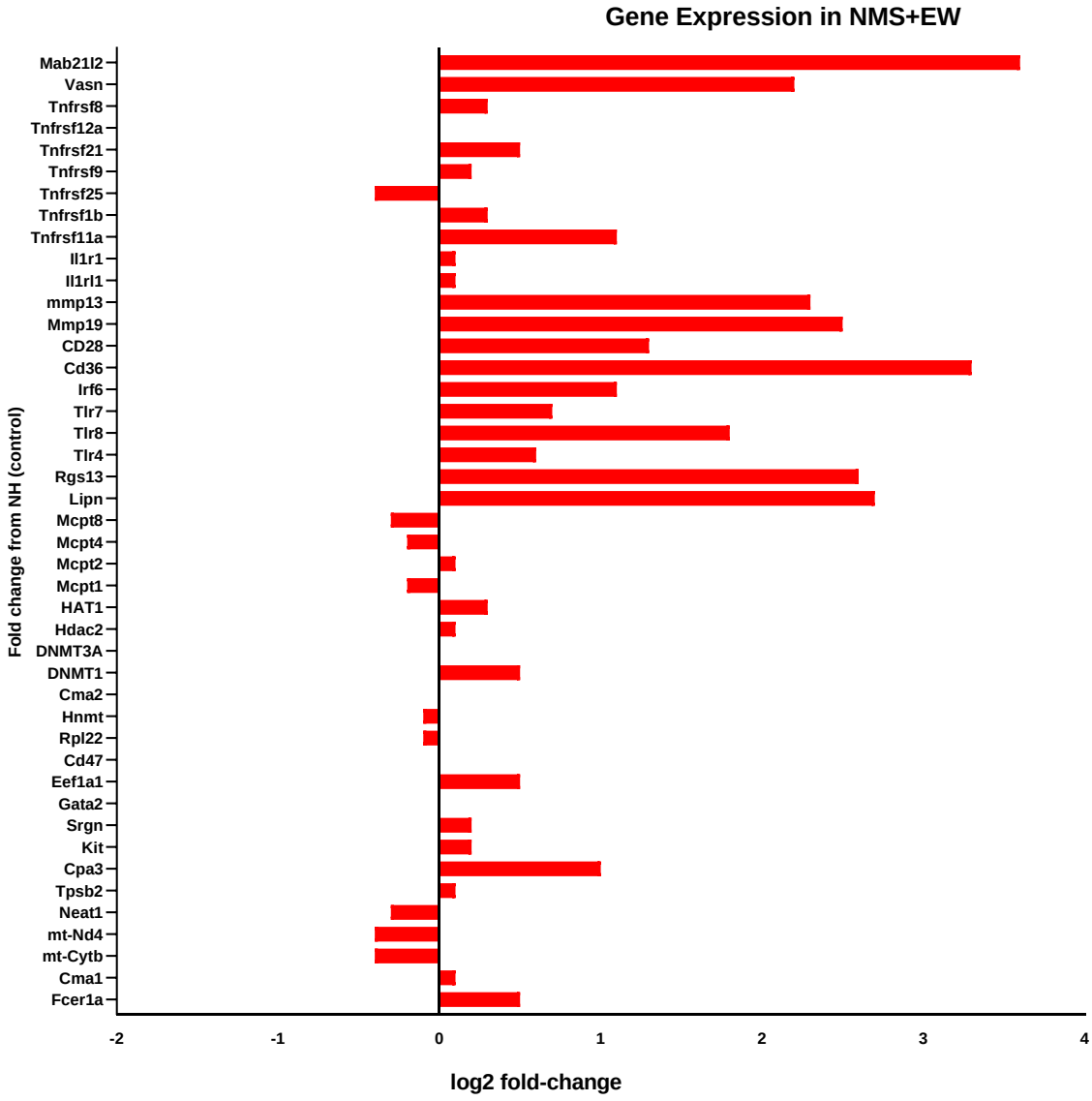


Figure 4.17 Gene expression of Mas Cell relevant genes in NMSEW BMMCs Relative to NH BMMCs.

Vasn	Vasorin, interactions between nervous system and vascular system
TLR4,7 and 8	Pathogen defense, inflammation
TNF Receptor Family	Pathogen defense, inflammation
Mcpt8,2	Proteases, tissue remodeling, repair and inflammation.
Mmp13 and Mmp19	Metalloproteinases, tissue remodeling, repair and inflammation
Irf6	Transcription factor, inflammation
Mab21l2	B-Cell responses, cell fate, cell proliferation
Rgs13	G-couple receptor mediated responses

Figure 4.18 Biological Relevance of Mast Cell Relevant genes in NMSEW BMMC Relative to NH BMMCs

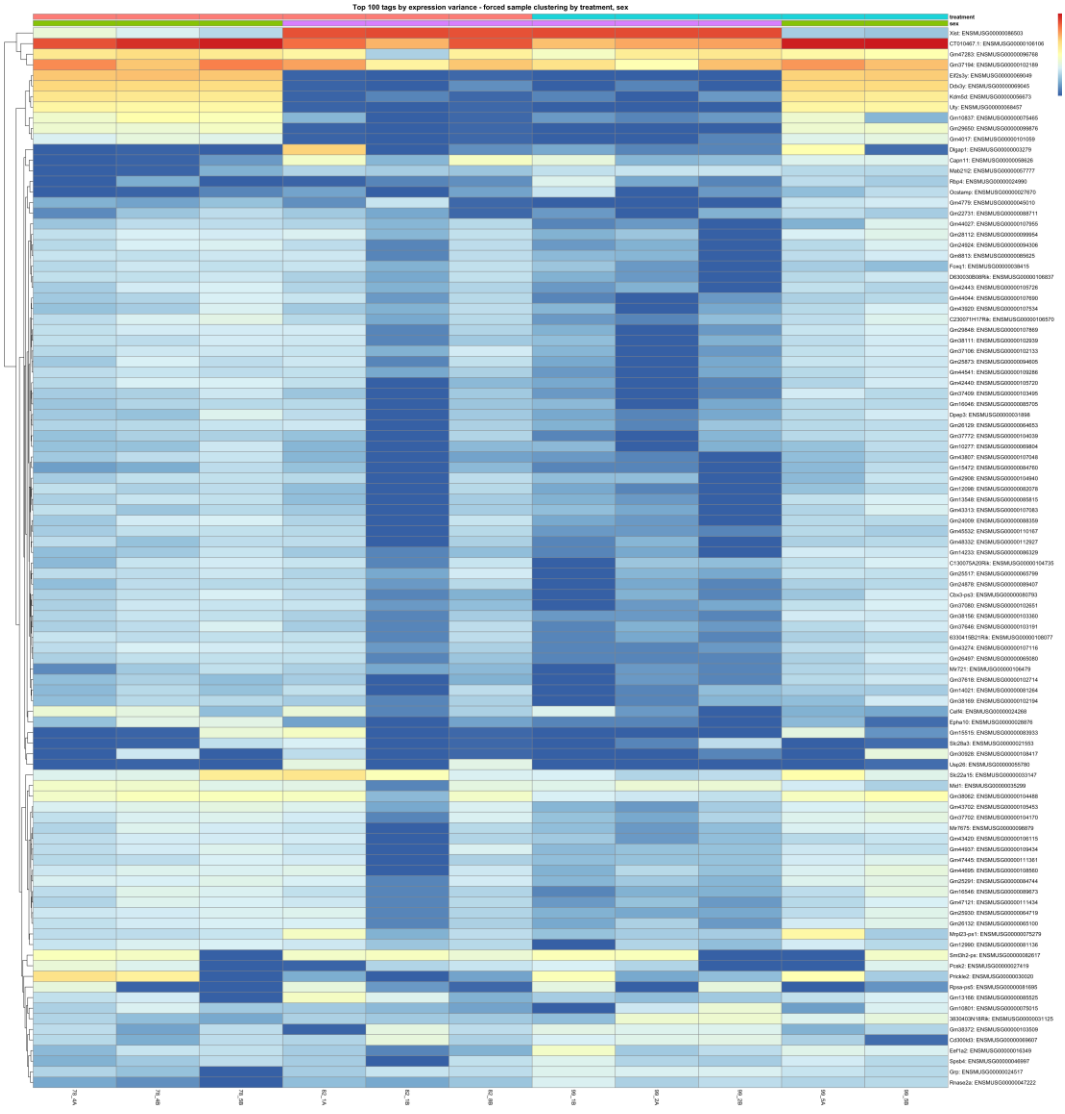


Figure 4.19 Heatmap Sample clustering by Treatment and Sex.

ELA induces upregulation of TLR4 and Increases Severity to Systemic LPS Administration

Given that our previous studies demonstrated an enhanced response of MCs to LPS challenge *in-vitro* and further transcriptome analysis identified an increase in *TLR4* gene expression. We then assessed TLR4 protein expression in NH and NMSEW BMMCs, and further subjected NH and NMSEW mice to an LPS challenge. Briefly, mice were injected with LPS (1mg/kg), rectal temperatures and clinical scores were monitored evert hour for 6 hours [28]. Our results indicate that NMSEW mice had an increase in basal MC protein expression of TLR4 **(Fig 4.20)**

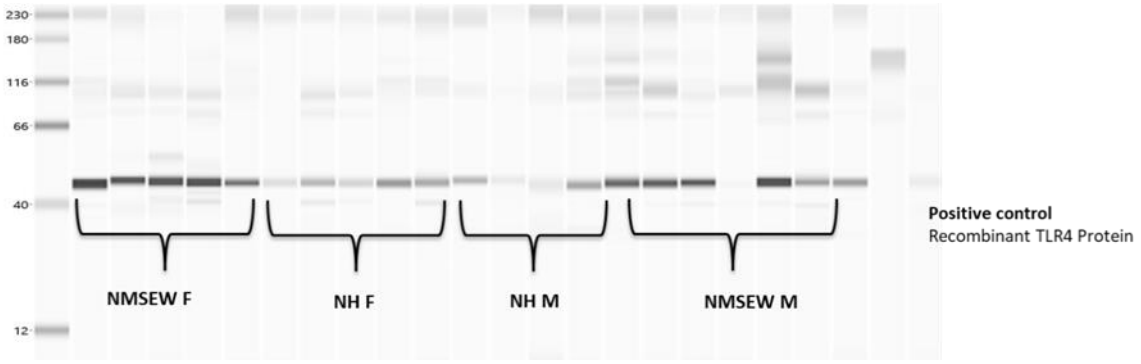
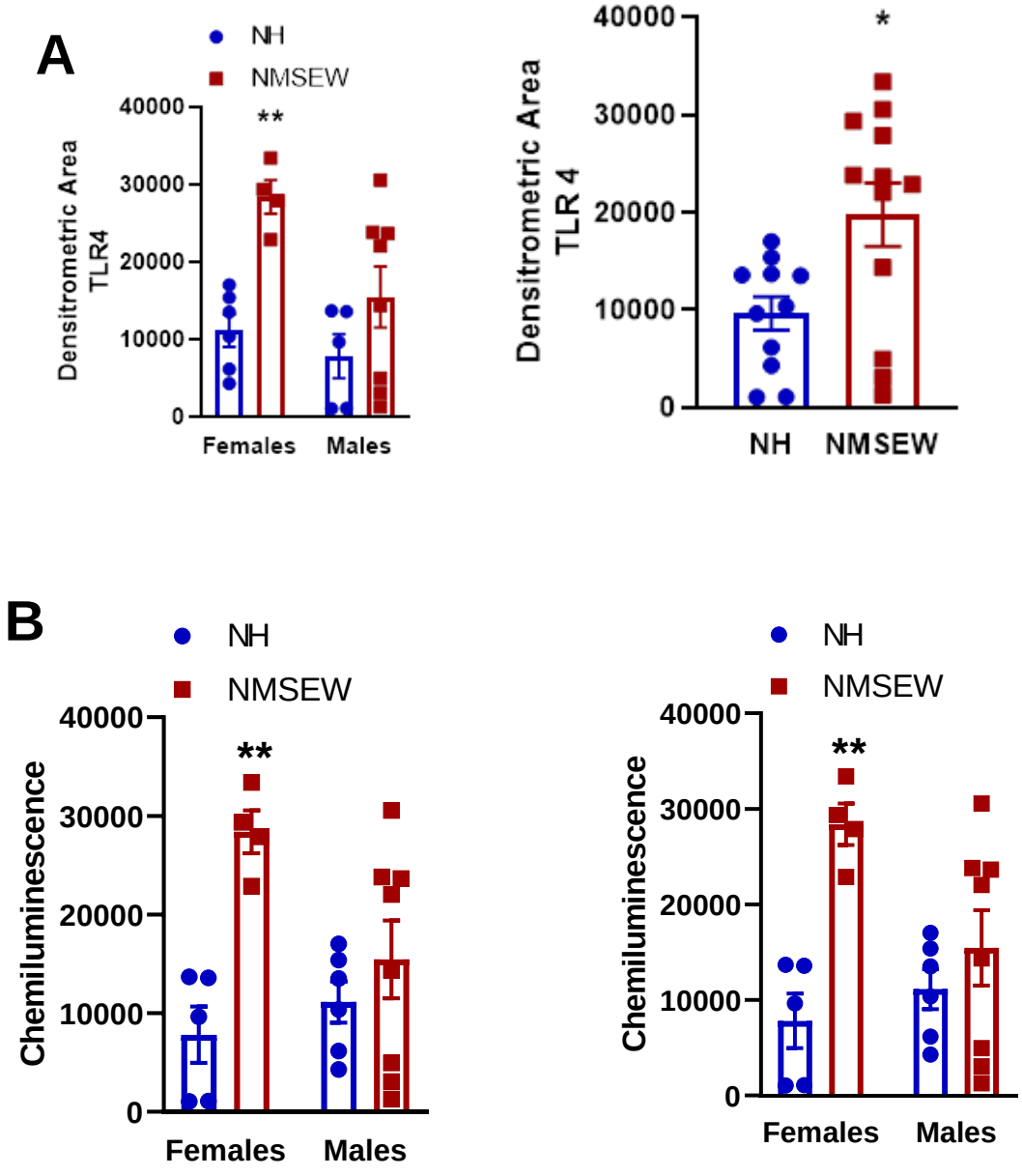


Figure 4.20 Capillary western blot (Wes) analysis of TLR4 in NH and NMSEW BMMCS.

Figure 4.20 (cont'd)



Capillary Western analyses were performed using the Protein Simple Wes System. **(A)** Densitometric area calculated with image J, **(B)** chemiluminescence calculated in GraphPad.

Figure 4.20 (cont'd)

Higher expression of TLR4 in NMSEW mice and when we split by sex its significantly higher in females. Unpaired Student's t-test. Data represent means SEM of a representative experiment with 6 bone marrow donors per treatment. *P<0.05, **P<0.01, ***P<0.0001.

Discussion

Maternal separation plus early weaning heightens MC activity in adult mice

Early life stress can have long-lasting consequences in behavior and physiology. In humans, early-life stressors such as disruptions of maternal-infant bonds have been a significant determinant on the development of behavioral disorders later in life, such as anxiety and depression [29]. Additionally, there is strong evidence showing the association between deprived rearing conditions and inflammatory markers later in life, such as increased IL6 and CRP in adults [30-32], thereby, increasing disease susceptibility later in life. In fact, Danese et al., 2007 reported twice CRP levels in children maltreated, increasing the risk for cardiovascular diseases in their early 20's. Importantly, Kiecolt and colleagues determined the long-term impact of ELA on cell aging, inflammation, and susceptibility to adult stress; in this study, multiple childhood adversities were associated with increased IL6 and TNFα levels in older adults; also, these individuals had a shorter telomere length which translated to a 7-15 years difference in lifespan compared to individuals without a history of childhood adversity.[33, 34]. These associations were exacerbated in adults having a caregiver position at that time, highlighting the impact of ELA on adult stress susceptibility.

Maternal separation is a well-known model of early life adversity. Maternal presence is vital in maintaining normal HPA axis responses [35-38]. No stress is expected during this period, and the HPA axis is in a quiescent form [39]. Early disruptions in the maternal-infant bond have shown to induce dysregulations in the HPA responsiveness, making it difficult for the system to return to basal unstressed conditions, producing long-lasting abnormal changes in cortisol levels [40]. Moreover, previous studies have demonstrated the permanent effects of ELA on immunological responses, with activation of inflammatory responses in the brain and periphery [41, 42]. Increased MC numbers in the brain and high susceptibility to allergies and encephalomyelitis, with increased neurotrophic mediators [43] have been shown in animals exposed to ELA [44, 45].

Maternal separation plus early weaning programs MC progenitors for enhanced degranulation, preformed mediator release and de novo synthesized mediator

Our study previously demonstrated that ELA prompted an increased MC tissue activation and exacerbated stressed-induced MC tissue activation, suggesting a programming effect of ELA on MC progenitors. Previous studies in our lab have also shown long-term increased GI symptom severity and MC numbers in mice and pigs subjected to models of early life adversity [9, 10]. However, it remains unknown if ELA induces changes in hematopoietic MC activity, altering MC's progenitors toward a hyperactive phenotype in adulthood. Since MCs are hematopoietically derived from the bone marrow, we later focused on BMMCs for our experiments. Our study demonstrated that BMMC from ELA-induced animals had increased histamine content and histamine and β-hexosaminidase release upon IgE stimulation. Additionally, we

demonstrated that ELA induced higher IL-3 induced proliferation in BMMCs originated from stressed animals. This finding is consistent with that of Heidt et al., 2014 who found that chronic social stress-induced changes in neurotransmitter levels in the bone marrow and this, in turn, increased proliferation of stem cells, leading them to an overproduction of neutrophils and monocytes [46]. Similarly, studies have found that chronic stress causes a redistribution of leukocytes, increasing granulocytes, and decreasing lymphocytes, possibly as an adaptive mechanism in response to the early stress induced [47, 48]. Although we do not know the exact mechanism of why MCs are increased or hyperactive, changes induced by ELA on the bone marrow environment niche may be responsible for the programming of MC progenitors.

Further, our study demonstrated that ELA provoked an increase in MC protease-1 and TNFα, an inflammatory cytokine produced by MCs known to play a role in acute stress inflammation, responsible for enhancing inflammatory responses [49]. Mast cells can release pre-stored and later synthesized TNFα [50], mMCP-1 is a granule protein released upon degranulation also involved in inflammatory processes. Our study also demonstrated an increase of IL6, TNFα and upon IL33 and LPS stimulations in BMMCs from ELA mice. This finding is in line with other studies indicating an increase in inflammatory cytokines and increased leukocyte inflammatory gene expression after subjection to chronic stress [45, 51-53]. Repeated social defeat in males has been known to increase the number of neutrophils and monocytes as well as induce elevated levels of IL6 and NGF [54]. Additionally, our RNA sequencing analysis of BMMCs derived from stressed (NMSEW) and controls (NH) mice demonstrated that, at baseline (unstimulated) conditions, there was an enhanced gene expression of immune and MC

relevant genes in NMSEW BMMCS relative to NH BMMCs. In our study, there were 2,093 differentially expressed genes in BMMCs from stressed mice, with 1,203 of these genes being different in NMSEW females compared to female controls indicating that females are more sensible to early life stress and they MCs progenitors seem to be more affected by it. Our principal component analysis identified that transcripts from NMSEW grouped differently from controls, and within the group NMSEW, females grouped independently. Sexual dimorphism in MCs has been previously demonstrated in our lab by Mackey et al., 2016 female MCs contain more mediator content and are more susceptible to activation upon immunological or psychological stressors; additionally, females show basal gene expression differences compared to their males counterparts [10, 55].

Maternal separation plus early weaning increases TLR family expression

Interestingly, in BMMCs from NMSEW mice, there was an upregulation of genes related to TLR signaling, specifically of genes that encode for TLR 4 and TLR8, this was exacerbated in NMSEW females. Also, receptors from the TNF family was upregulated in NMSEW BMMCs. MCs, contain TLR1-9; among these, signaling through TLR 2 and TLR4 are implicated in danger and bacterial recognition [56, 57]. Upon TLR activation, there is a release a number of cytokines and leukotrienes, including potent cytokine TNFα and IL6, crucial for an effective immune response against pathogens. Specifically, TLR4 in MCs was found to be critical for the production of necessary pro-inflammatory cytokines and leukocyte recruitment needed to protect against acute peritonitis. [58, 59]. Models of early life stress have previously demonstrated to induce higher pro-

inflammatory cytokines, and NFKB signaling [56]. Also, repeated exposure to stress increased NK activity [45] and increased viral infection rate[60].

Potential causal drivers of MC changes in NMSEW mice

Early exposure to inflammatory signals has been shown to have a long-term impact on immune function, inducing long-lasting phenotypic changes in macrophages [61]. The immune system is stimulated and triggered by the stress signals released during ELA. Stromal cells, leukocytes, and hematopoietic cells are known to express β-adrenergic receptors, and signaling through these receptors can heighten leukocyte responses and affect progenitors in the bone marrow [62]. Progenitor cells are able to sense danger signals, such as toll-like receptor ligands, or neurotransmitters [63-66]. Similarly, Yañez et al.,2013 demonstrated that early TLR signaling in the hematopoietic niche influences myelopoiesis and macrophage function[67]. Vascular cell adhesion proteins and selectins are responsible for keeping the HSC in niches. Stromal cells including, mesenchymal cells, epithelial cells; osteoblasts secrete stimulating cytokines for example, M-CSF, GM-CSF, CXCL12 that bind to receptors on HSCs triggering a change in migration, proliferation and lineage differentiation.

MCs originate from a multipotential progenitor different form the common myeloid progenitor[68], however other already committed cells can be reprogrammed into the MC lineage by altering the expression of early developments transcription factors. Retrovirally transduced committed lymphoid precursors with transcription factor GATA-2 is able to convert basophils into MC precursors [69]. Interestingly GATA-2 came up as a top upstream regulator in our NMSEW BMMC transcriptome analysis. GATA-2 transcription factor has known to promote chromatin accessibility MC enhancer regions, promoting transcription of genes and antigenic stimulation [70]. In contrast MC

differentiation is negatively regulated by expression of y CCAAT/ enhancer-binding protein alpha (CEBPα) [69]. Other important transcription factors playing a role in early MC development are GATA-1, PU.1 and the microphthalmia-associated transcription factor (Mitf). Mitf is important as it regulates a number of MC receptors, proteases and signaling molecules critical for MC function and phenotype [71]. Additionally, there are different isoforms of Mitf, controlled by MC-regulatory elements. Identification of the different isoforms and sequences as well as the main targets will provide insight into the regulatory networks in MCs [72-74].

The most known pathway of MC activation is the IgE-Ag activation; it has been shown that PKC plays a major role in the signaling cascade. PKC activation induced the AP-1 transcription factor [75]. AP-1 is composed of several jun and fos family of proteins which can homodimerize or Heterodimerize with other jun or fos proteins to form the AP-1 complex. IgE-Ag stimulated MCs display an increase in the AP-1 mRNA levels[76]. The complex networks of AP-1 proteins are involved in the transcriptional control of MCs. Other groups have defined the existence of other AP-1 complex, comprised of different proteins [77]. A critical player in the signal transduction of many cytokines is the JAK-STAT pathway, this transcription factor is critical for a complete mast cell activation including degranulation and cytokine production [78, 79]. To add to the complexity chromatin modifications interfering with the binding of transcription and co regulators ultimately influencing gene expression play a major role in MC differentiation, development phenotype and function, few studies have explored the role of epigenetic modification in regulating MC phenotype and responses [80, 81]. Understanding the different regulatory networks in MC differentiation and development

provides important aspects into the mechanisms of stress-induced MC phenotypic changes and can provide targets to treat stress-induced MC related disorders in the future.

Conclusions

Early life adversity plays a significant role in the development and exacerbation of inflammatory disorders later in life. The present study demonstrates that ELA has a long-term impact on bone marrow-derived progenitor MCs, programming them toward hyperactivity in adulthood. MCs harvested from the femurs of mice exposed to ELA at a young age exhibited increased mediator content, release, and increased pro-inflammatory cytokines upon stimulation and increased proliferation rate. Additionally, these studies provide evidence that ELA induces transcriptional changes in BMMCs, altering MC immune relevant genes and pathogen recognition receptors, potentially as a long-term adaptation to stress, but with enduring deleterious consequences. Up until now, the literature was vague regarding the impact of ELA on specific immune cell types. Together, these data provide a new, more in-depth understanding of the effects of ELA on MC phenotype and function and how this may be playing a role in later inflammatory disease susceptibility. Further elucidation of the mechanisms driving early stressed-induced MC hyperactivity will help develop future intervention strategies.

APPENDIX

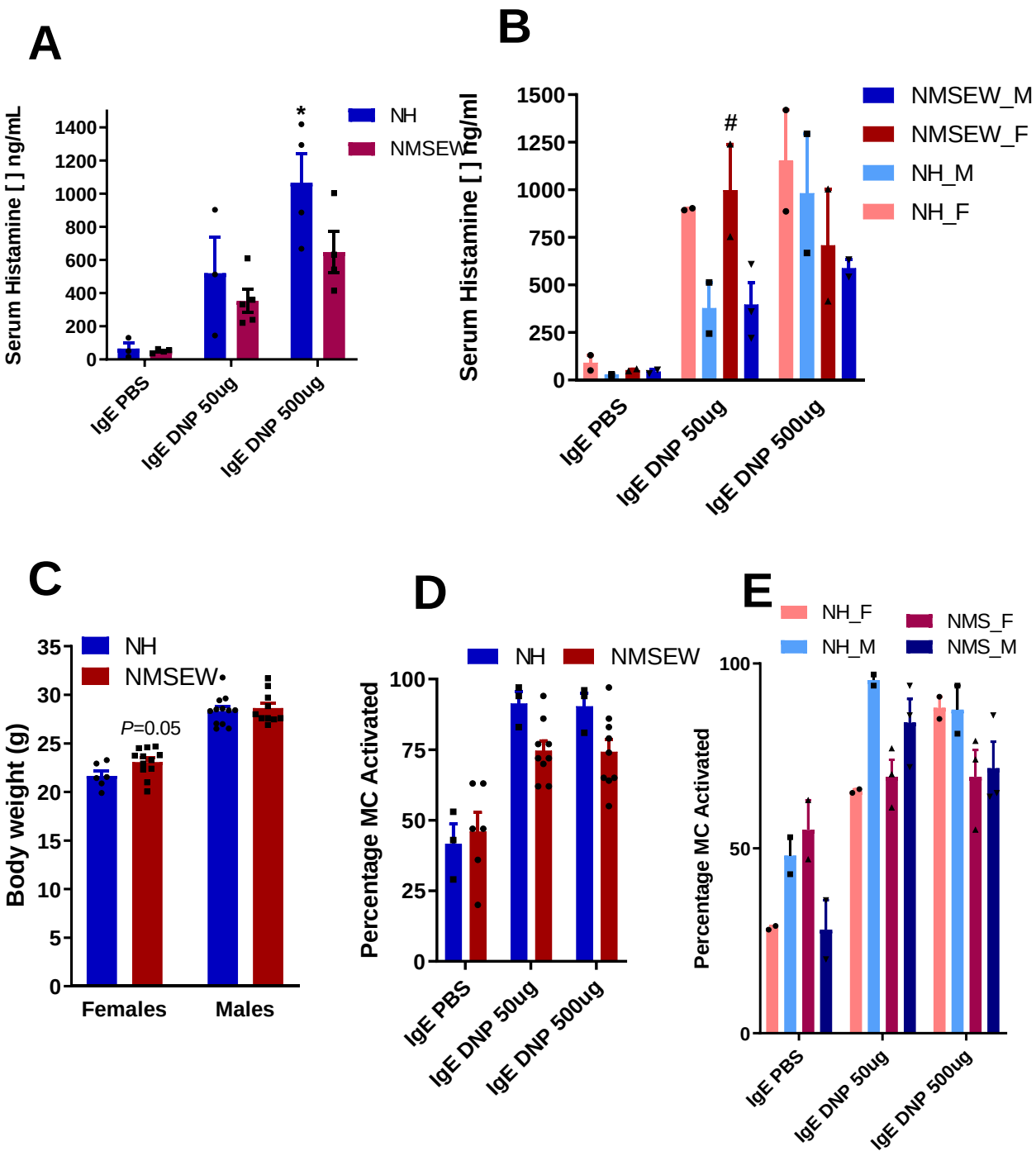
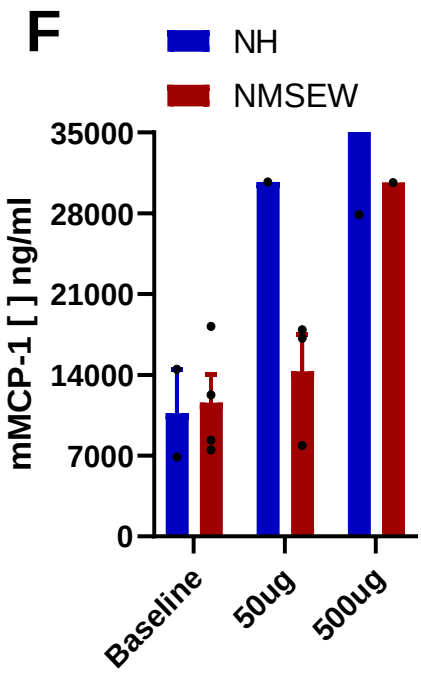


Figure S.1 Passive systemic anaphylaxis in NH and NMSEW mice.

Figure S.1 (cont'd)



Mice were sensitized with anti-DNP monoclonal IgE. Twenty four hours later they were injected via i.p. with the antigen DNP to induce PSA. Serum was later collected to evaluate histamine and mast cell protease-1 concentrations. Mesenteric windows from jejunum were collected to evaluate MC tissue activation. A) Serum histamine levels at 30 minutes post-challenge. B) Sex differences in serum histamine levels at 30 minutes post-challenge. C) Sex differences in body weight of NH and NMSEW mice. D) Percentage of MC activation in mesenteric windows of jejunum after DNP challenge. E) Sex differences in percentage of MC activation in mesenteric windows of jejunum after DNP challenge. F) Mast cell protease-1 levels at baseline and post-IgE challenge.

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CHAPTER FIVE

Summary

Overview

There is increasing concern for the numbers of child abuse and neglect in US alone. There is a lot evidence to support the vital role that ELA plays in neuronal and immunological regulation, consequently leading to the development of inflammatory diseases later in life. With these studies, we add to the literature demonstrating the impact that ELA exerts on immune cell activation, specifically how ELA affects MC responses. Since increased MC activation was observed in adult mice that had previously undergone our ELA protocol of maternal separation plus early weaning, we focused on the impact of ELA on bone marrow derived mast cells.

In *Chapter 3*, we demonstrated that mice exposed to ELA exhibited increased MC activity into adulthood. Further, we demonstrated that ELA increased MC number and activation in mesenteries of jejunum, additionally; ELA also increased basal serum histamine levels. Moreover, ELA increased the total histamine content in peritoneal MCs compared to NH controls, validating the ELA BMMCS phenotype observed in tissue and the high basal serum histamine levels in ELA animals.. Interestingly, ELA increased serum histamine levels in NMSEW mice following adult chronic stress as well as increased fecal pellet output, intestinal permeability and increased MC activation in mesenteries of jejunum. Although we did not find significance in the levels of mMCP-1 after subjection to adult chronic stress, NMSEW females had a higher release of mMCP-1 after subjection to adult chronic stress. Additionally, in Chapter 3, we explored the impact of ELA on MC activation upon subjection to adult immunological stress, for this, early life-stressed mice and controls were subjected in adulthood to a MC-dependent model of PSA. We found that NMSEW mice had increased PSA-induced

hypothermia at 30 min post injection. Unfortunately we did not find a difference in histamine concentration or mMCP-1 levels at 30 minutes post injection, suggesting an earlier time point for collection is needed to assess differences in MC mediator release. Moreover, we found that NMSEW mice exhibited increased clinical scores induced by PSA compared to controls at 15 minutes post-injection, suggesting 15 minutes post-injection is a good time point to evaluate MC mediators and activation in the future. These findings support other studies linking early life stress to heightened immune responses. Furthermore, these studies demonstrate that our ELA model of maternal separation plus early weaning is a good model to assess the effects of ELA on MC activation and mediator release. This chapter demonstrated that ELA is induced long-term changes on MC progenitors, programming them toward a hyperactive phenotype in adulthood and thereby exacerbated MC responses in adulthood both at basal levels and also upon subjection to psychological and immunological stress..

In *Chapter 4*, because of our previous findings of ELA programming MC progenitors toward a hyperactive phenotype in adulthood, we explored the effects of ELA on bone marrow-derived mast cells (BMMCs) from adult mice that had been previously exposed to our ELA protocol. We observed that BMMCs derived from NMSEW mice exhibited increased pre-stored histamine content, and higher pre-stored TNFα cytokine release also. Further, NMSEW BMMCS had higher β-hexosaminidase and histamine release upon IgE-DNP stimulation. We also evaluated other non-IgE stimulation to assess if the heightened responses observed were specific to IgE-DNP stimulation. We stimulated BMMC with IL33, and LPS and assess cytokine release after stimulation. BMMCs derived from NMSEW mice exhibited higher IL6 release into

supernatant upon IL33 stimulation and also exhibited higher TNF and IL6 release into supernatant upon LPS stimulation and this response seems to be exacerbated in NMS EW females.

To evaluate if there were differences in the proliferative capacity of BMMCs, we assessed MC proliferation by BrdU incorporation. Our results indicate that early life stress-induced a higher IL-3 induced proliferative effect in the bmmcs. We did not find any differences in the expression of c-*kit* and FceRI receptors. Also, there were no morphological differences between NH and NMSEW BMMCs upon visualization with toluidine blue stain. Suggesting differences in mediator release observed is not due expression of receptors or morphological changes. Moreover in Chapter 4 we wanted to gain more insight into the global gene expression changes induced by ELA on bone marrow MC progenitors, for this, we profiled gene expression patterns by RNA sequencing on BMMCs derived from our NH controls and BMMCs derived from our early life stressed mice (NMSEW). We demonstrated that ELA induced transcriptional changes in BM MC-progenitors. More than 2000 genes were differentially expressed between NH and NMSEW BMMCs; also RNA sequencing revealed that 1,203 genes were altered by ELA in males and 890 genes in females. Transcriptome analysis of NH and NMSEW BMMCS revealed enhanced gene expression of immune related genes *TLR4*, *TLR7*, *and TLR8 and* TNF receptor family in NMSEW BMMC. Additionally, transcriptome analysis showed increased gene expression of MC related genes such as proteases and metalloproteinases; such as Mcpt4, *Mcpt8*, and *Mcpt2, Mmp13, Mmmp19,* which are involved in tissue remodeling, repair, and inflammation. Interestingly irf6, Mab2112 were increased in NMSEW BMMCs, genes involved in the

regulation of inflammatory cytokines. Also observed in NMSEW BMMCS was g\higher gene expression of *Lipn and vasn*, genes involved in lipid metabolism and vascular system. Given that transcriptome analysis revealed an increase in TLR4 expression in NMSEW BMMCs, we then evaluated TLR expression in BMMCS and confirmed our previous RNA seq data, finding an increase in TLR 4 protein expression in BMMCs derived from NMSEW mice, this increase was significantly higher in females NMSEW BMMCs. Interestingly LPS came up as a top gene regulator in females and males, as seen in Chapter 3 BMMCs had a higher response to LPS challenge, particularly females. Also we found Gata2, TP53, and ERBB2 as top regulators. Given the heightened in-vitro responses of NMSEW BMMCs to LPS and the increased TLR4 expression we then evaluated LPS responses in-vivo. We found an increase in serum TNFa cytokine release in NMSEW animals upon LPS injection. Although we had a small number of animals for his experiment further studies are needed to elucidate the biological significance of possessing increased TLR and TNF receptor family in NSMEW mice.

Together, these findings demonstrate robust long-term changes in MC activity and responses induced by ELA, this correlates with the literature showing the long-lasting immune changes induced by stress. Additionally, these studies demonstrate the significant role that MC play in potentially aggravating stress-induced inflammatory disorders later in life.

Highlight of Novel Findings

Early life adversity programs MC progenitor cells for increased proliferative capacity, enhanced preformed mediator and de novo synthesized mediator release in adulthood and increases susceptibility to chronic psychological stress and Immunological stress in adulthood.

These findings have significant implications for understanding how early life stress increases stress and inflammatory disease susceptibility later in life. Previous studies evaluating the effects of early life stress focus solely on behavioral outcomes and neurological disease susceptibility. Few studies have focused on the impact of early life stress on adult immune function and long-term disease susceptibility. Importantly, these findings are the first to report that ELA programs MCs toward a hyperactive phenotype in adulthood. The data presented here demonstrates that tissue resident MCs from mice previously exposed to ELA had increased preformed mediator content and that these early life stressed animals have increased serum histamine levels in adulthood. Importantly, the data presented here demonstrated that ELA exacerbates MC responses upon subjection to adult psychological stress and immunological stress. These findings highlight the significance and importance that ELA could be playing in promoting long-term chronic inflammatory diseases. Little is known about the effects of ELA on MC activity and function. Notably, the data presented here demonstrated a programming effect of ELA on bone marrow-derived MCs. Prominently, this is the first time that has been shown that bone-marrow derived MCs from adult early life stressed mice possessed higher mediator content and increased responses to various stimuli.

Additionally, progenitor MCs from early life stressed animals had increased proliferative capacity compared to non-stressed controls. To determine the best causal relationship between early life stress, mediator content and release in MCs, and proliferative capacity, these parameters have to be measured over time as well as the characterization of the transcriptional changes that take place from the application of stress into adulthood. Although it is well studied that stress plays a significant role in the development and exacerbation of several diseases, these findings suggest that immune development and function are controlled, influenced, and programmed by stress signals released during an early traumatic event, increasing disease susceptibility later in life. More importantly that ELA has the ability to influence the hematopoietic niche of MC progenitors shaping them toward hyperactivity in adulthood. This may be due potentially as an adaptive mechanism in response to stress rescuing and enhancing the first line of defense in preparation for future stressful or infectious encounters. However, more studies are needed in the future to elucidate the mechanisms by which ELA affects MC activity and disease susceptibility. Determining the mechanism by which stress affects the immune system will help develop therapeutic strategies that will help target and limit MC activity in stress-induced MC related disorders.

Early life adversity increased TLR4 expression in bone marrow derived MC progenitors and LPS-induced hypothermia in adulthood.

Despite knowing the debilitating behavioral health effects of having early childhood adversity little is known about the immunological consequences that ELA can have on MC progenitor cells. The data presented here demonstrate that ELA is capable of

inducing transcriptional changes in BM MC progenitor cells. These findings are the first to report that ELA increased the expression of the TLR receptor family as well as the TNF receptor family, key players in the immune response against infections. Moreover, these findings are the first to report differentially expressed genes in females and males upon subjection to ELA. Notably TLR4 was significantly upregulated in NMSEW animals, these was confirmed by measuring proteins levels in MCs. This is significantly important as MCs respond to TLR ligands by secreting mediators and inflammatory cytokines such as IL6 and TNFa, which in our studies were also found increased in our NMSEW animals. In addition it is known that TLR ligands can synergize with FceRI signaling enhancing inflammatory responses. Also, our transcriptome analysis showed LPS as a top regulator in NMSEW BMMCs and increased NF-KB signaling pathway, this pathway is important in mediating immune responses and is part of the cascade triggered by TLR signaling. Our in-*vivo* LPS challenge demonstrated an increased in LPS-induced hypothermia in NMSEW mice.

Together these studies demonstrate the long-term impact ELA can have inflammatory disease susceptibility, particularly in TLR signaling. Further studies are needed to elucidate the precise mechanism and timing by which ELA increases TLR4 expression and the positive and negative biological consequences of possessing this heightened receptor and hence increased immune responses.

Limitations and Future Directions

A limitation of this study is the inability to isolate the mechanism by which early life adversity is programming mast cell hyperactivity. Further characterization of mast cells epigenome and transcriptome is needed to identify the extent and pin down the signaling pathways early life stress influences mast cell activity. Additional engraftment experiments using $Kit^{W\text{-}sh/W\text{-}sh}$ mice and transplanting them with the NMSEW BMMC hyperactive phenotype to elucidate additional roles in biological responses. Limited use of transgenic approaches that will allow specific activation or inhibition of MC mediators and determine the possible involvement of pathways in the stress-induced phenotype.

Future directions should focus on site-specific-epigenetic and genetic alterations induced by early life stress on bone-marrow-derived mast cells. For instance, determine the epigenetic modifications, and changes in methylation patterns of promoter regions of pro-inflammatory cytokines in BM-derived MCs, to investigate if ELA alters the epigenetic profile to present pro-inflammatory cytokines. For instance, Janusek et al., 2017 found a reduction in DNA methylation of the IL-6 promoter region which was associated with childhood trauma in African American men [1]. This, in turn, increased stress-induced IL-6 production.

Further exploration of the regulators responsible for accommodating the epigenetic changes related to stress will be very promising. Looking into the top stream regulators found in our transcriptome data, for instance, GATA2, TCL1A, and TP53 and their potential immunological consequence, characterization of immune profiles of knock

downs during and after ELA and the biological significance of this may help reveal the underlying mechanisms and help develop interventional strategies in the future.

Further full characterization of the impact of early life stress on other MC receptors is needed. For instance, the investigation of the impact of early life stress on toll-like receptors signaling pathways and its biological significance. It remains unclear how or why ELA induces upregulation of toll-like receptors, further transcriptome analysis of LPS challenged BMMCs is needed to elucidate potential gene targets that will help elucidate basic mechanisms underlying inflammation. Understanding how ELA affects LPS responses in vivo by performing in vivo studies of LPS challenge in mice where MCs are ablated will provide fruitful information about its role in ELA and infection. In future studies, primary human mast cells from adults with a severe history of early life adversity should also be evaluated and assessed their history of infection recurrence to associate the role of the impact of ELA on toll-like receptor programming.

Determine the timing by which early life stress shifts the activation trajectory of MC progenitor cells. Determine the extent of this MC phenotype and imprinting; for instance, perform transgenerational studies to determine if the offspring of mice exposed to early life adversity shows the same MC hyperactivity and epigenetic marks.

Our work here highlights the significant impact that ELA has on MC phenotype and function and role in long-term-inflammatory disease susceptibility. Future work should focus on elucidating the timing and mechanism and by which ELA impacts bone –marrow-derived MC development, function, and gene expression. Finding a

mechanism and target will help develop therapeutic strategies to alleviate stress-induced mast cell-related diseases.

REFERENCES

REFERENCES

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