Peripheral Blood Biomarkers and Experimental Autoimmune Encephalomyelitis (EAE)

A Thesis in
Anatomy
by
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Abstract

Multiple sclerosis (MS) is a demyelinating autoimmune disease. Current diagnostic methods for MS require measures including magnetic resonance imaging or analysis of cerebral spinal fluid for the initial diagnosis as well as for prognosis. There is a need to identify non-invasive markers that correlate with the onset and progression of MS, as well as a patient’s response to therapy. This research utilized mouse models of experimental autoimmune encephalomyelitis (EAE), relapse-remitting type (RR-EAE) and chronic progressive type (CH-EAE), to examine total leukocyte number and as well as the distribution of leukocyte subtypes as biomarkers that may correlate with different stages of MS. Previous studies have shown that modulation of the OGF-OGFr axis with either exogenous OGF or LDN changes the course of clinical disease and neuropathology in mice with RR-EAE or CH-EAE. Thus, changes in leukocyte number and distribution following treatment of mice with either opioid growth factor (OGF) or low dosages of naltrexone (LDN) were monitored. Increases in total leukocyte number as well as neutrophil and eosinophil levels were found at disease onset and peak disease for mice with RR-EAE or CH-EAE. There were increases seen in the leukocyte and neutrophil levels for the groups treated with OGF and LDN in the CH-EAE and RR-EAE models, respectively. In summary, total leukocyte number and distribution of leukocytes change with the onset of EAE, whereas neutrophil and eosinophil levels fluctuate with modulation of the OGF-OGFr axis. These changes suggest that analyses of blood samples may provide a non-invasive tool for monitoring the progression of MS and its response to treatment.
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List of Abbreviations

ANOVA- Analysis of variance
C – Celsius
CBC – Complete Blood Count
CD – Cluster of Differentiation
CFA – Complete Freund’s Adjuvant
CH-EAE – Chronic Experimental Autoimmune Encephalomyelitis
CSF – Cerebral Spinal Fluid
CXCL1 - C-X-C motif ligand 1
CXCL16 – C-X-C motif ligand 16
CXCL 5 - C-X-C motif ligand 5
° - Degrees
EAE – Experimental Autoimmune Encephalomyelitis
ELISA – Enzyme Linked Immunosorbent Assay
IFA – Incomplete Freund’s Adjuvant
IL - Interleukin
kg - Kilogram
LDN – Low Dose Naltrexone
Met-Enk – Methionine Enkephalin
mg - Milligram
MHC – Major Histocompatibility Complex
mm - Millimeter
MOG – Myelin Oligodendrocyte Glycoprotein
MRI – Magnetic Resonance Imaging
MS – Multiple Sclerosis
MTB - Mycobacterium tuberculosis
MW – Molecular weight
NK – Natural Killer
NMSS – National Multiple Sclerosis Society
μL – Microliter
ng - Nanogram
OCT – Optimal Cutting Temperature
OGF – Opioid Growth Factor
OGFr – Opioid Growth Factor receptor
PBS – Phosphate Buffered Saline
% - Percent
PLP – Proteolipid Protein
PPMS – Primary Progressive Multiple Sclerosis
PTX – Pertussis toxin
RPM – Rotations per minute
RR-EAE – Relapse Remitting Experimental Autoimmune Encephalomyelitis
RRMS – Relapsing-Remitting Multiple Sclerosis
SAP – Serum Amyloid P
SPMS – Secondary Progressive Multiple Sclerosis
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Chapter 1: Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a debilitating neurological disorder that affects more women than men and usually has an onset between the third and fifth decades of life (Sospedra and Martin, 2005). Multiple Sclerosis is characterized as either relapse-remitting (RRMS) or progressive, depending on the course of the disease (NMSS, 2014). The most common form of MS affecting approximately 85% of those diagnosed with the disorder is RRMS. Patients spend periods of time in remission, and then symptoms return, with this cycle becoming shorter and more frequent until the patient relapses into progressive MS. Due to the lack of a single diagnostic test for MS, several methods to detect the disease have been proposed over the last few decades (Poser et al., 1983) including brain scans via magnetic resonance imaging (MRI) to observe the lesions of the central nervous system (McDonald et al., 2001) and the reduced importance of immunoglobulin G and oligoclonal bands in the cerebral spinal fluid (Polman et al., 2011). Multiple Sclerosis is an autoimmune disorder, which classically has been associated with the CD4+ T cells. Recently research on the immune cells in MS and EAE, the mouse model of MS, has revealed that CD8+ T cells and B cells are also important markers for progression of the disorder (Sospedra and Martin, 2005). However, no single diagnostic tool suffices for detection and classification of this neurological disease.

1.2 Current Treatments for MS
There are a number of medications for the treatment of MS that can be classified into three different types (NMSS, 2014). The first is palliative medications, which alleviate the symptoms of multiple sclerosis and its flares. The second type of medication shortens the length of relapses or flares, and includes corticosteroids. These drugs are usually prescribed at the time of a flare and are taken in high dosages for short duration. The third type of medication modifies the overall progression of multiple sclerosis, and these drugs include: Copaxone, Betaserone, Rebif, Aubagio, or Avonex. Several of the disease modifying medications are categorized as interferon beta medications that increase the amount of interferon beta in the patient, which has been shown to decrease antigen levels and suppress Th1 cells, but the exact mechanism remains unknown (Minagar, 2013). Another disease modifying medication, fingolimod (Gilenya), is a sphingosine 1-phosphate receptor modulator that blocks lymphocytes from crossing the blood brain barrier and entering the central nervous system, thus the lymphocytes remain in the peripheral lymph nodes (NMSS, 2014). Natalizumab (Tysabri) is a humanized mouse monoclonal antibody that prevents leukocytes from crossing the blood brain barrier (Minagar, 2013). Dimethyl fumarate (Tecfidera) also prevents leukocytes from crossing the blood brain barrier (Minagar, 2013; NMSS 2014). Glatiramer acetate (Copaxone) is a subcutaneous injection that mimics the myelin basic protein (NMSS, 2014). Copaxone is a random arrangement of the four amino acids: L-tyrosine, L-glutamate, L-alanine, and L-lysine that imitates myelin basic protein and binds to the MHCII of the T-cells (Minagar, 2013; NMSS, 2014), and prevent the immune system from targeting the myelin surrounding the spinal cord.

1.3 Blood Cells and Functions
Blood cells can be classified into two different groups: red blood cells or erythrocytes and white blood cells or leukocytes. The erythrocytes are responsible for the transport of oxygen and carbon dioxide to and from the different tissues of the body. The leukocytes constitute the immune system for the blood. There are several different types of leukocytes including, neutrophils, eosinophils, basophils, monocytes and lymphocytes (Ross and Pawlina 2011). Lymphocytes are categorized based on the function of the cell and include B-cells, T-cells, and NK-cells; many of these immune cells are studied in the EAE disease models and in MS. Lymphocytes are responsible for multiple functions, such as antibody production, production of regulatory molecules called interleukins, and cell mediated immunity (Latimer et al., 2003). Monocytes are large leukocytes that are responsible for phagocytosis of damaged tissues and cells, as well as other foreign matter in the body (Latimer et al., 2003). In addition to their phagocytic action, monocytes also secrete cytokines and other chemicals associated with inflammatory responses.

Eosinophil number often increases in patients having allergic reactions, chronic inflammation, and anaphylaxis (Ross and Pawlina 2011; Latimer et al., 2003). Basophils are the least common of the leukocytes. These cells are also associated with allergic reactions. Finally, neutrophils are the most common of the leukocytes in humans (Ross and Pawlina 2011). Neutrophils are the first of the leukocytes to respond to damage and frequently migrate along with monocytes and lymphocytes into connective tissue or epithelium. This migration of neutrophils is only from the circulating blood to the target tissue, not from the tissue to the blood (Latimer, et al., 2003). Like monocytes, the neutrophils have a phagocytic function, mainly targeting bacteria. Neutrophils have a
rapid turnover rate, where the neutrophil population in the blood is replaced around two and half times a day.

Leukocytes can be measured by a complete blood count (CBC) (National Institute of Health, 2012). This test will give the total levels of erythrocytes and leukocytes, as well as other aspects of the blood, namely hematocrit and hemoglobin levels. This test can indicate the presence of autoimmune diseases, allergic responses, dehydration, and other such conditions. However, the CBC cannot differentiate between the types of leukocytes. In order to differentiate between the leukocytes, a blood differential test must be conducted (National Institute of Health, 2013).

Recent studies have given some insight into the functions of these immune cells in MS and its disease model EAE. Rumble et al. has shown that in the EAE model, there is a large increase in circulating neutrophils and monocytes prior to the onset of disease (2015). In addition to this increase in neutrophils and monocytes, it was shown that there is an upregulation of the cytokine CXCL1 and granulocyte colony-stimulating factor. As a transition from the EAE to MS, it has been shown that there is an increase in a similar cytokine, CXCL5, and this cytokine has been correlated to disease severity and the number of MS lesions.

In addition, previous research has yielded some insights into interleukin levels and other cytokines during the progression of EAE. It has been shown that the interleukin IL-22 is increased at the onset of disease and that IL-21 has been decreased at peak disease (Murta and Ferrari 2013). In addition to these chemokine levels, it has been shown that specific neutrophils, polymorphonuclear neutrophils with CXCR2, have been
linked to such events in the formation of EAE as: demyelination, activation of peripheral lymphocytes and monocytes, and degradation of the blood brain barrier.

1.4 OGF-OGFr Axis

Endogenous opioids have been shown in previous studies to have a role in the immune system and in cell proliferation (Zagon, et al., 2009). One such endogenous opioid is opioid growth factor (OGF), or as it is chemically termed, [Met$^5$]-enkephalin (Zagon, Verderame, and McLaughlin, 2002). OGF has an inhibitory effect on cell proliferation, but does not induce apoptosis, or necrosis (McLaughlin and Zagon, 2012). OGF acts on the cyclin-dependent inhibitory kinases that regulate the cell cycle. The upregulation of these cyclin-dependent kinases by OGF prevents the transition into the S phase of the cell cycle, thereby halting proliferation of the cell. OGF is tonically active, reversible, and can bind to receptor in normal and abnormal cells. The receptor for OGF, opioid growth factor receptor (OGFr), presents characteristics that are similar to other compounds that bind to opioid receptors: antagonistic action of naloxone and stereospecific binding. OGFr is found on the nuclear envelope of cells (Zagon et al., 2002).

An antagonist to the OGF-OGFr axis is naltrexone (McLaughlin and Zagon 2012). Naltrexone is a small compound (377 MW) that binds to OGFr, as well as other classical opioid receptors, preventing the binding of endogenous molecules, such as OGF. In low doses, the naltrexone blockade lasts 4 to 6 hours. This blockade causes a biofeedback mechanism and the production of more OGF, which will bind to OGFr in the 18 to 20 hours following the blockade. This biofeedback mechanism from LDN results in
an inhibitory effect on cell proliferation. The same biofeedback mechanism occurs following high doses of naltrexone, however due to the longer blockade from the high dose, the OGF cannot bind to OGFr, and the paradigm results in an accelerated cell proliferation (McLaughlin and Zagon, 2012).

1.5 Modulation of the OGF-OGFr Pathway and EAE

Previous studies on EAE and mice treated with either OGF or LDN have shown that OGF inhibits disease progression and disease severity in both forms of EAE, RR-EAE and CH-EAE (Hammer et al., 2013; Rahn, McLaughlin, Zagon, 2011; Campbell et al., 2012). In a recent study whereby mice with RR-EAE were treated with OGF at the time of disease induction, animals had fewer relapses than those that did not receive OGF (Hammer, et al., 2013). Mice treated with OGF had on average less than 0.5 relapses, while mice treated with saline had an average of 2 relapses. Moreover, several mice displayed no relapse following the initial onset of the disease. In addition, mice treated with OGF entered remission beginning four days after disease onset, with sixty-two percent of the mice treated with OGF in remission fourteen days following disease onset. In contrast, the mice treated with saline had only one mouse that entered remission. Thus, the mice treated with OGF had a total of 16.5 days in remission, compared to the mice treated with saline, which had a total of 3 days in remission.

In studies on MOG-induced CH-EAE, where mice were treated with either LDN or OGF, only 63 % of the mice treated with LDN showed behavioral symptoms, and 68% in the group of mice treated with OGF (Rahn et al., 2011). Mice treated with OGF or LDN had reduced behavioral scores compared to the mice treated with saline; 3.2-times
lower scores for OGF and 2.6-times lower scores for LDN. In another study on the CH-EAE model, the mice that were given OGF showed a 48% decrease in behavioral scores after 20 days of treatment and a 36% decrease after 40 days of treatment (Campbell et al., 2012). In addition to the behavioral scores, the mice that were treated with OGF also had a decrease in percent demyelination. The mice treated with just saline had 13.8%, 11.8% and 13.8% demyelination on days 10, 20, and 40 respectively. In contrast, the OGF treated mice had only 8.6%, 3.2%, 7.5% demyelination.

1.6 Goal and Hypothesis

While the treatments for MS have been improving, the diagnosis for the disorder is still imprecise, due to a lack of a singular diagnostic test (McDonald et al., 2001). In addition, current diagnostic methods of lumbar punctures and MRIs are inconvenient and not cost effective. Even when correctly done, a lumbar puncture could damage a lumbar spinal root. More over, MRIs can be uncomfortable to the patient, as well as time consuming and expensive. The hypothesis of the research is that changes in leukocyte number and distribution of eosinophils, neutrophils and/or lymphocytes may provide a correlation with the progression of MS/EAE, and response to treatment. While a singular diagnostic test may not be determined, this study aims to observe whether changes in the complement of immune cells in the peripheral blood in mice correlate with disease severity and response to therapy. An overall goal is to establish a new diagnostic method for MS that is non-invasive and requires only a small sample of blood.
Chapter 2: Methodology

2.1 Mice

The RR-EAE studies utilized female SJL/JOrlCRL mice from Charles River Laboratories (Wilmington, MA USA) (Hammer et al., 2013), and the CH-EAE study used C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA). All mice in the study were between 5-8 weeks of age and were group housed in conventional open top cages in a room isolated from other mice. All mice were given constant access to food and water. Hydrogel and food were provided on the cage floor if the mice were unable to access the food and water because of severe disease. Mice were euthanized if both hind limb and forelimb were paralyzed. All protocols are in accordance with the guidelines set by the National Institute of Health and were approved by the Pennsylvania State University College of Medicine’s Institutional Animal Care and Use Committee, protocol 2006-055.

2.2 Induction of Disease

The RR-EAE disease model was induced with PLP using published protocols (Miller et al., 2007; Hammer et al., 2013, 2015). Each mouse received three subcutaneous injections of an emulsion of PLP139-151 and complete Freund’s adjuvant (CFA) totaling 300 µL. Mycobacterium tuberculosis combined with incomplete Freund’s adjuvant to make the CFA. Following the subcutaneous injections of PLP, each mouse received two intraperitoneal injections of 200 ng pertussis toxin (PTX) dissolved in PBS, one immediately following the PLP injections, and one two days later. A small subset of
mice received subcutaneous injections of PBS instead of the emulsion and intraperitoneal injections of PBS instead of the PTX and was considered the control.

The CH-EAE disease model was induced by three injections of an emulsion of MOG$_{35-55}$ and CFA, totaling 200 µL (Miller et al., 2007). The emulsion was injected subcutaneously on Day 0 post disease induction with a second injection of CFA at Day 7 post disease induction. In addition, the mice received two intraperitoneal injections of 200 ng PTX dissolved in PBS on day 0 post disease induction and day 2 post disease induction. A small subset of mice received subcutaneous injections of PBS instead of the emulsion and PTX and was considered the control.

2.3 Treatment of Disease

Treatment for each mouse was initiated at the time of established disease. Following two separate observations of behavioral symptoms within a twelve-hour period, mice were randomly assigned into treatment groups, and assigned to either saline treatment or LDN for the RR-EAE study, or OGF for the CH-EAE study. Mice in each group received daily injections of 0.1 µl the respective treatment, either PBS for the saline treated group, 10 mg/kg OGF, or 0.1 mg/kg LDN.

2.4 Behavioral Observations

Disease severity was monitored daily by behavioral observations, beginning on the day that behavioral symptoms are first observed. Behavior was scored on a scale from zero to ten, with zero indicating no disease, and ten indicating death from disease. Behavioral parameters that were measured: normal tail, distal tail drag, tail dragging but with tone, tail with no tone, normal gait, slightly wobbly gait, moderately impaired gait,
severely impaired gait, normal righting reflex, slow righting reflex, righting reflex absent, limb strength normal, limb faulting, limbs near paralysis, and limbs paralyzed (Hammer et al., 2013, 2015; Campbell et al., 2012).

2.5 Blood Collection

Blood was collected from the mice using a survival collection method, to allow for multiple samples to be collected from the same mouse as the disease progressed. This was accomplished by using a 5.5 mm Goldenrod animal lancet to pierce the submandibular vein. Up to 100 µl of blood was collected in a heparinized capillary tube and transferred to an Eppendorf tube for centrifugation. Several drops were collected from the Eppendorf tube for blood smears, prior to centrifugation of the blood sample.

Blood collection for the RR-EAE studies occurred at the following points during the disease progression: baseline or prior to disease induction, disease onset or approximately day nine post disease induction, peak disease or approximately day fourteen post disease induction, remission and the termination of the study. At the remission collection point a small subset of mice were euthanized. Blood Collection for the CH-EAE study occurred at the following points during disease progression: baseline or prior to disease induction, disease onset, peak disease, and the termination of the study.

2.6 Cytology

Blood smears were stained with a Quik Diff 3 step stain (Azer Scientific). Slides were examined using an Olympus BH2 microscope at 10X objective (total magnification 100X) with ocular grids. Total leukocytes per field, as well as eosinophils, neutrophils, monocytes, and lymphocytes were counted within 4 fields per mouse, and 5 mice per
group at each time point. The percentage of eosinophils, neutrophils, monocytes, and lymphocytes within a 200-cell field was recorded.

2.7 Tissue Collection

Mice were euthanized either at remission or termination of the study. Following euthanasia, a blood sample was collected from each mouse by a terminal cardiac puncture. Blood was transferred to an Eppendorf tube for centrifugation and a small sample of blood was used to make a blood smear. Following the terminal blood collection, a section of spinal cord was taken from the mice at the level of the lumbar enlargement. The spinal cords were removed by extrusion through the cervical end of the spinal column. Spinal cord samples were snap frozen in isopentane and embedded in OCT compound.

2.8 Histopathology

Embedded spinal cords were sectioned on a cryostat at -20°C. Sections were cut to a thickness of 10 µl. Slides were post fixed in a combination of acetone and 95% ethanol at -20°C. Spinal core sections were stained for astrocytes with GFAP and for mast cells with Toluidine blue (Korkmaz et al., 2014; Campbell et al., 2013). The observations were conducted at 20X magnification. Images of the spinal cord were taken near the central canal, with both white matter and grey matter being imaged. Mast cells and activated astrocytes were counted using Image Pro Plus 6.2 software.

2.9 Western Blot
A western blot was performed on the serum samples using a 12% SDS-PAGE gel with a 6% stacking gel. The samples compared were a non-diseased mouse and a RR-EAE mouse at disease onset. A known protein sample was used as a control. Gels were transferred to nitrocellulose blots and then stained with Ponceau stain to observe the bands of protein.

2.10 Statistical Analysis

Behavioral data and differential cell count data was analyzed using Prism Graphpad 5 software. Behavioral data were analyzed by calculating the mean and standard error for each treatment group for each day, followed by two tailed t-tests. Cell counts were averaged for each blood smear, and for each mouse. Differential cell data counts were analyzed with both t-tests and one-way analysis of variance (ANOVA) followed by Tukey post-tests to determine significant differences between treatment groups.
Chapter 3: Normal Mice and RR-EAE Mice

3.1 Study Structure and Hypothesis

The goal of the first study was to determine changes in the peripheral blood that occurred over the course of RR-EAE, and to correlate these changes with clinical behavior scores in comparison to non-diseased, normal mice. Mice immunized to present with RR-EAE were utilized because more than 85% of the patients with MS have RRMS (NMSS 2014). The hypothesis for this study is that RR-EAE will result in leukocyte distribution that differs from that in normal mice, and that may correlate with clinical disease progression.

Normal and RR-EAE mice were administered phosphate-buffered saline on a daily basis. Blood samples were collected at the following time points: baseline, disease onset, peak disease, disease remission, and termination of the study, with the addition of a relapse category.
3.2 Behavior

Figure 3.2E presents clinical behavior scores of mice in the RR-EAE group receiving saline and non-diseased controls. Disease onset occurred approximately nine days post induction of PLP\textsubscript{139-151}. The RR-EAE mice had a mean behavioral score of 1.3 ± 0.5, representing an average score over a twelve-hour period following the first sign of disease. The signs of disease onset for this study consisted of a slightly wobbly and impaired gait, hind limb faults and slowed righting reflex. These behavioral findings were indicative of the onset of the disease. The non-immunized control mice had no signs of disease.

Peak disease in the RR-EAE group occurred at fifteen days post induction of PLP\textsubscript{139-151} (Figure 3.3E) when the RR-EAE mice had a mean behavioral score of 4.4 ± 0.5. Peak disease was accompanied by tail dragging, slow righting reflex, either slightly impaired gait or moderately impaired gait, and hindlimb foot faults, and/or hindlimb and forelimb faults. The non-disease control mice again showed no signs of disease.

At approximately day 22 post induction of PLP\textsubscript{139-151}, or at disease termination on day 28 post induction of PLP\textsubscript{139-151}, the RR-EAE entered into a disease remission, which is characterized by a reduction of disease score from the peak disease. For remission, a mouse must display a reduction in disease score if is less than 1.5 for two consecutive days (Figure 3.4E). At the time of remission, RR-EAE mice had mean clinical disease scores of 3.1 ± 0.1, a 29.5% decline in behavioral severity. The signs of disease
remission for this study were a slightly impaired gait, tail dragging, slow righting reflex, and hind limb faults.

If the mouse showed an increase of 2 points in behavioral score, then it was considered to be in relapse (Figure 3.5E). The RR-EAE mice showed an average behavioral score of 4.6 ± 0.2. The signs of disease relapse for this study were a moderately impaired gait, tail dragging, slow righting reflex, and hindlimb faults. The non-disease control mice had no remission or relapse.

3.3 Total Leukocytes

Blood was collected prior to the onset of RR-EAE to serve as a baseline comparison. Figure 3.1A displays the baseline levels for both the RR-EAE and CH-EAE levels. The baseline levels are similar between the RR-EAE and CH-EAE groups, suggesting that SJL and C57Bl/6J mice have comparable baseline leukocyte levels, prior to the induction of disease. Mice in the RR-EAE group had a mean of 56.8 ± 4.7 leukocytes whereas mice in the CH-EAE group had a mean of 60.7 ± 8.9 leukocytes per field.

Total leukocyte levels for the RR-EAE at disease onset are presented in Figure 3.2A. The RR-EAE mice had a higher total leukocyte level than the non-diseased control. The non-diseased control mice had a mean total leukocyte level of 69.2 ± 3.9 leukocytes per field, while the RR-EAE mice had a mean of 243.1 ± 10.46 leukocytes per field. This is an increase of 251%, with a P-value of <0.0001 at 95% confidence.

Figure 3.3A shows the total leukocyte number for the RR-EAE mice at peak disease. The non-disease mice had a mean total leukocyte level of 91.6 ± 9.9 leukocytes
per field, while the RR-EAE group had a mean total leukocyte level of $125.1 \pm 12.7$ leukocytes per field. This is an increase of nearly 37%; however, these values did not differ significantly.

Figure 3.4A demonstrates total leukocyte number at remission. The non-diseased mice had a mean total leukocyte level of $71.5 \pm 2.6$ leukocytes per field in comparison to RR-EAE mice with a mean total leukocyte level of $48.2 \pm 7.5$ leukocytes per field, a significant (P<0.01) decrease of 40%. Figure 3.4D shows the average behavioral scores for the different groups of mice.

Mice were considered in a state of relapse if following a remission, their behavior score increased. Figure 3.5A shows the total leukocyte levels at disease relapse. The non-diseased control mice had a mean total leukocyte level of $50.5 \pm 4.7$ leukocytes per field, while the RR-EAE mice had a mean total leukocyte level $37.0 \pm 3.7$ leukocytes per field. This was a decrease of 29%; however it was not statistically significant. Figure 3.5D displays the average behavioral scores of the different groups.

### 3.4 Differential Leukocytes

Percentages of neutrophils at disease onset are presented in Figure 3.2B. The non-diseased control mice had a mean of $3.7\% \pm 0.5\%$ of the total leukocytes, while RR-EAE mice had a mean of $12.0\% \pm 2.9\%$ of the total leukocytes representing over a three-fold increase (P<0.01).

Figure 3.2C shows the percentages of eosinophils at disease onset. The non-diseased control mice had a mean of $1.7\% \pm 0.4\%$ of the total leukocytes. The RR-EAE
mice had a mean of 1.6% ± 0.2% of the total leukocytes. This difference is not significant.

Lymphocyte levels for disease onset are presented in Figure 3.2D. The non-diseased control mice had a mean of 59.1% ± 3.2% of the total leukocytes, while the RR-EAE mice had a mean of 48.7% ± 4.8% of the total leukocytes. The differences between the groups are not significant. Refer to Figure 3.2E for the average behavior scores for disease onset.

The differential leukocyte levels at peak disease are described in Figure 3.3B and Figure 3.3C. Figure 3.3B presents the neutrophils at peak disease. In comparison to non-diseased control mice that had an average of 3.4% ± 0.8% of the total leukocytes, mice in the RR-EAE group had a mean neutrophil level of 16.3% ± 1.4% of the total leukocytes, representing an increase of approximately 5-fold. These data were significantly different at P<0.0001.

Figure 3.3C displays the eosinophil levels at peak disease. The non-diseased control mice had a mean eosinophil level of 2.3% ± 0.6% of the total leukocytes. The RR-EAE mice had a mean eosinophil level of 4.0% ± 0.4% of the total leukocytes. This is an increase of almost 74% and was significantly different between the groups.

Lymphocyte levels for peak disease are shown in Figure 3.3D. The non-diseased control mice had a mean lymphocyte level of 64.4% ± 8.0% of the total leukocytes. Similar to the control mice, the RR-EAE mice had a mean lymphocyte level of 56.2% ± 5.5% of the total leukocytes. These values did not differ.
The average behavioral scores of mice at peak disease are presented in Figure 3.3E.

Neutrophil number at disease remission is shown in Figure 3.4B. The non-diseased control mice had a mean neutrophil level of 5.5% ± 1.4% of the total leukocytes. The RR-EAE mice had a mean neutrophil level of 20.6 ± 4.7% neutrophils per field. This is an increase of more than 3 fold. Statistical evaluation revealed that these values differed significantly at P<0.01.

Eosinophil number at disease remission is presented in Figure 3.4C. The non-diseased control mice had a mean eosinophil level of 2.8% ± 0.2% of the total leukocytes. The RR-EAE mice had a mean eosinophil level of 3.6% ± 1.0% of the total leukocytes, which was twice the percentage reported for non-diseased control mice. Statistical evaluation revealed that the means differed significantly at P=0.02.

Figure 3.4D describes the lymphocyte levels at disease remission. The non-diseased control mice had a mean lymphocyte level of 48.8% ± 3.3% of the total leukocytes. The RR-EAE mice showed similar percent lymphocytes. Average behavioral scores at the time of remission are presented in Figure 3.4E.

Neutrophil distribution for mice in relapse is shown in Figure 3.5B. Non-diseased control mice had a mean neutrophil level of 9.5% ± 1.3% of the total leukocytes, in comparison to mice with RR-EAE that had a mean neutrophil level of 20.4% ± 3.9% of the total leukocytes. This increase was more than double the non-diseased control and the two groups differed significantly at P<0.003.
Evaluation of mice in relapse revealed that the percent of eosinophils for RR-EAE mice (2.3% ± 0.3%) is half of the non-diseased mice (4.2% ± 1.0%) (Figure 3.5C). This difference was shown to be significant (P=0.02). The number of lymphocytes was significantly depressed in RR-EAE mice by 30% from the non-diseased control mice level of 63.4% ± 2.8% of the total leukocytes (Figure 3.5D); these values were significantly different at P<0.0001. Behavioral scores at the time of relapse are reported in Figure 3.5E.

For all collection points, there were no significant differences for the percent monocytes. The mean percent monocytes similar and did not exceed 0.17%.
Baseline and Disease Onset Comparison

Figure 3-1 Comparison of Total Leukocyte Number at Baseline and Disease Onset in both RR-EAE and CH-EAE. A) Baseline total leukocyte levels were observed prior to disease induction of RR-EAE and CH-EAE in the SJL and the C57Bl/6 mice, respectively (n= 4, n=3). B) Disease onset for both RR-EAE and CH-EAE models. The disease onset collection point occurred on days 9 and 10 for the RR-EAE and CH-EAE models, respectively. Each symbol represents a single blood smear. Significantly different from corresponding Normal mice at p<0.001 (***, +++).
Figure 3-2 Differential Blood Cell Count and Average Behavioral Scores at Disease Onset for Mice with RR-EAE. Disease onset occurred on day 9 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for 5 mice in the normal and RR-EAE groups. Symbols represent averages for each smear. Significantly different from values for Normal mice at P<0.05 (*) or P<0.001 (**). (E) The average behavioral score for the RR-EAE mice at disease onset was 1.3 ± 0.5.
Figure 3-3 Differential Blood Cell Count and Average Behavioral Score for RR-EAE at Peak Disease. Peak disease occurred on day 15 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for 5 mice in the normal and RR-EAE groups. Symbols represent averages for each smear. Significantly different from the Normal group at $P<0.05$ (*) or $P<0.001$ (**). E) The average behavioral score for the RR-EAE mice at peak disease was $4.4 \pm 0.5$. 
Disease Remission

**Figure 3-4. Differential Blood Cell Counts and Average Behavioral Score at Disease Remission.** Disease remission occurred on day 22 post disease induction, and corresponded to study termination. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocyte (D) for 5 mice in the normal group and 9 mice in the RR-EAE groups. Symbols represent averages for each smear. Significantly different from values for Normal mice at P<0.05 (*), P<0.01 (**). (E) The average behavioral score at disease remission for the RR-EAE mice is $3.1 \pm 0.1$. 

![Graphs showing differential blood cell counts and average behavioral score at disease remission.](image-url)
Disease remission occurred on day 28 post disease induction, and corresponded to study termination. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for 5 mice in the normal group and 9 mice in the RR-EAE groups. Each symbol represents averages for each blood smear. Significantly different from values for Normal mice at $P<0.05$ (*), $P<0.01$ (**), or $P<0.001$ (***)

(E) The average behavioral score for the RR-EAE mice at disease relapse is $4.6 \pm 0.2$. 

**Figure 3-5 Differential Blood Cell Counts and Average Behavioral Score for Disease Relapse.**
Chapter 4: RR-EAE with LDN Treatment

4.1 Study Structure and Hypothesis

This study expanded on data described in Chapter 3 with the addition of treatment with LDN. Naltrexone is an opioid antagonist, acting on both classical and non-classical opioid receptors. In low doses, the naltrexone causes a short-term blockade of the interaction between endogenous opioids, including OGF, and their receptors (McLaughlin and Zagon, 2012). This short-term blockade of the OGFr causes a biofeedback mechanism to increase levels of endogenous OGF following the blockade. Previous studies with EAE and LDN have shown that there is a reduction in disease severity in the CH-EAE model (Rahn et al., 2011). The hypothesis of this study is that differential cell counts in the peripheral blood may be altered by treatment with LDN.

For this study, mice with RR-EAE were randomly assigned at disease onset to groups receiving either saline or LDN. Behavior was observed daily. Blood was collected at the following time points: baseline, disease onset, peak disease, disease remission, and disease relapse.
4.2 Behavior

The onset of disease was described in Chapter 3.2 and Chapter 3.3. At disease onset, only the non-diseased control and RR-EAE groups were present. Following disease onset, the RR-EAE mice were randomly assigned into saline and LDN treatment groups. As with Chapter 3, the non-disease control mice had behavioral scores of zero at all time points.

Figure 4.1E presents the average behavioral score for peak disease, which occurred on day fifteen post PLP\textsubscript{139-151} induction. The RR-EAE mice treated with saline had a mean behavioral score of 5.3 ± 0.8 and mice in the RR-EAE group treated with LDN had a mean behavioral score of 4.5 ± 1.0.

Figure 4.2E shows the average behavioral score for disease onset. Disease remission occurred on day 23 post PLP\textsubscript{139-151} induction or at disease determination. The RR-EAE mice treated with saline had a mean behavioral score of 3.3 ± 0.2. The RR-EAE mice treated with LDN had a mean behavioral score of 2.5 ± 0.4.

Disease relapse occurred at day 32 post PLP\textsubscript{139-151} induction. At this point, mice in the RR-EAE group treated with saline had a mean behavioral score of 3.0 ± 0.0, as did mice receiving LDN. It was noted that very few mice in this study actually went to into a disease relapse; only one saline treated RR-EAE mouse, and two LDN treated RR-EAE mice displaying clinical relapse.

4.3 Total Leukocytes
Figure 4.1A presents the total leukocyte levels for the RR-EAE groups at peak disease. The non-disease mice had a mean total leukocyte level of $99.4 \pm 14.4$, while the RR-EAE group treated with saline had a mean total leukocyte level of $56.8 \pm 14.2$, a decrease of nearly 43%. The RR-EAE group treated with LDN had a mean total leukocyte count of $75.4 \pm 7.5$, a decrease of 24%. The differences between the groups were not significant.

Figure 4.2A shows the total leukocyte levels at disease remission. The non-diseased mice had a mean total leukocyte level of $102.8 \pm 5.1$, while the RR-EAE mice treated with saline had a mean total leukocyte level of $53.1 \pm 5.5$. This is a decrease of almost 48%. The RR-EAE mice treated LDN had a mean total leukocyte level of $49.1 \pm 9.1$, a decrease of nearly 52% over the non-disease control. Statistical analysis of the data revealed that the groups are significantly different at $P<0.0001$, and post-hoc evaluation showed that significant differences were noted between the non-diseased control and the RR-EAE mice treated with saline and the RR-EAE mice treated with LDN. Figure 4.2D shows the average behavioral scores for the different groups of mice.

Total leukocyte number (Figure 4.3A) at disease relapse revealed that non-diseased control mice had a mean total leukocyte level of $58.2 \pm 9.2$, whereas the RR-EAE mice treated with saline had a mean total leukocyte level of $100.8 \pm 18.3$. RR-EAE mice treated with LDN had a mean total leukocyte level of $65.1 \pm 11.5$ representing a 12% over the non-diseased control and a 35% decrease from saline-treated EAE mice. Figure 4.3D displays the average behavioral scores of the different groups.

4.4 Differential Leukocytes in RR-EAE Mice Treated with Saline or LDN.
Neutrophil percentages (Figure 4.1B) at peak disease demonstrates that non-diseased control mice had a mean percentage neutrophil of $8.2\% \pm 1.6\%$ in comparison to $18.8\% \pm 4.0\%$ for mice with RR-EAE receiving saline, an increase of more than two-fold. The RR-EAE mice treated with LDN had a mean neutrophil level of $17.3\% \pm 3.3\%$. ANOVA evaluation determined that the difference between the means is not significant.

Figure 4.1C displays the percentages of eosinophils at peak disease. The non-diseased control mice had a mean eosinophil percentage of $2.5\% \pm 0.3\%$, similar to the RR-EAE mice treated with saline that had a mean eosinophil percentage of $2.3\% \pm 0.9\%$. Mice in the RR-EAE group treated with LDN had a mean eosinophil level of $3.4\% \pm 0.7\%$, which demonstrates a significant reduction in the percentage of leukocytes that are eosinophils. An ANOVA determined that the difference between the means is not significant ($P=0.17$).

Lymphocyte percentages for peak disease are displayed in Figure 4.1D. Non-diseased control mice had a mean percent lymphocyte of $89.3\% \pm 1.6\%$. The RR-EAE mice treated with saline had a mean percent lymphocyte of $68.0\% \pm 13.7\%$ and the RR-EAE mice treated with LDN had a mean percent lymphocyte of $57.6\% \pm 9.0\%$. An ANOVA with a $P$ value of 0.01 revealed that the differences between the groups are significant, with the significant differences between the non-disease control mice and the LDN treated mice. The percent monocytes for all treatment groups at peak disease did not exceed 1% ($0.26\%$ for the control mice, $0.25\%$ for the saline treated RR-EAE mice and 0.0% for the LDN treated RR-EAE mice), and the difference between the means was found to be not significant. Refer to Figure 4.1E for the average behavioral score for peak disease.
The number of neutrophils at disease remission (4.2B) reflects that non-diseased control mice had a mean percent neutrophil of approximately 11%. The RR-EAE mice treated with saline had mean percent neutrophil of approximately 22%. RR-EAE mice treated with LDN had a mean percent neutrophil of approximately 22%. Statistical evaluation with ANOVA and post-hoc tests revealed that these means did not differ significantly. However, the differences between the non-diseased control mice and the RR-EAE mice treated with LDN were significant.

Figure 4.2C shows the eosinophil percentages at disease remission. The non-diseased control mice had a mean of 2.7% ± 0.3% of total leukocytes, whereas mice in the RR-EAE group receiving saline had a mean of 3.9% ± 0.9% of total leukocytes. However, RR-EAE mice treated with LDN had a mean of 2.0% ± 0.4% of the total leukocytes. Differences in eosinophils were not significantly decreased.

Lymphocyte percentages for disease remission are displayed in Figure 4.2D. The non-diseased control mice had a mean lymphocyte percentage of 86.1% ± 1.3%. The RR-EAE mice treated with saline had a mean lymphocyte level of 68.6% ± 11.1%. This is a decrease of 25% over the control mice. The RR-EAE mice treated with LDN showed a further decrease in lymphocyte levels, with a mean lymphocyte level of 57.0% ± 6.0%, a 23% decrease. An ANOVA with a P value of <0.05 determines that the difference between these groups is significant. The posttest revealed the differences between the RR-EAE mice treated with LDN and non-disease controls is significant.

The percent monocytes were shown to be significantly different at disease remission. The non-disease control mice and the LDN treated RR-EAE mice had
comparable mean percent monocytes (0.15% and 0.0%, respectively). The RR-EAE mice treated with saline showed significantly increased percent monocytes over the non-diseased control mice and the LDN treated RR-EAE mice. Refer to Figure 4.2E for the average behavioral score for disease remission.

Figure 4.3B shows the neutrophil percentages disease relapse. The non-diseased control mice had a mean percent neutrophil of 14.3% ± 2.4%. The RR-EAE mice treated with saline had a mean percent neutrophil of 22.1% ± 3.5. This is an increase of almost 185%. The RR-EAE mice treated with LDN had a mean percent neutrophil level of 17.1% ± 1.7% this is an increase of almost 59% over the non-disease control, but a decrease of almost 44% over the RR-EAE mice treated with saline. An ANOVA with a P-value of P=0.32 revealed that the difference between the groups is not significant.

Figure 4.3C shows the eosinophil percentages at disease relapse. The non-diseased control mice had a mean percent eosinophil of 1.8% ± 0.4%. The RR-EAE mice had a mean percent eosinophil of 1.0% ± 0.4%. The RR-EAE mice treated with LDN had a mean percent eosinophil of 3.3% ± 0.8%. An ANOVA with a P-value of P=0.1814 revealed that the differences between the groups is not significant.

Lymphocyte percentages at disease relapse are displayed in Figure 4.3D. The non-disease control mice had a mean percent lymphocyte of 83.6% ± 2.8%. The RR-EAE mice treated with saline had mean percent lymphocytes of 57.0% ± 4.9%. The RR-EAE mice treated with LDN showed a decrease of 45% over the control mice, with mean percent lymphocytes of 46.0% ± 5.6%. An ANOVA with a P-value of P<0.0001 revealed that the difference between the groups is significant. The post-test revealed that the
differences between the non-diseased control mice and the RR-EAE mice, both saline-treated and LDN-treated. The percent monocytes for disease relapse did not exceed 0.3% of the total leukocytes for all three treatment groups, and was shown to be not significant.

Behavior at disease relapse is shown in Figure 4.3E.
Figure 4-1 Differential Cell Count and Average Behavioral Score for RR-EAE with LDN Treatment at Peak Disease. Peak disease occurred at day 15 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for Normal mice (n=2), and those in the RR-EAE groups receiving either saline (n=2) or LDN (n=4). Symbols represent averages for each smear. (E) The RR-EAE + Saline mice has a mean clinical behavior score of $5.3 \pm 0.8$ and the RR-EAE + LDN mice had a mean behavior score of $4.5 \pm 1.0$. 
Figure 4-2 Differential Cell Count and Average Behavioral Score for RR-EAE with LDN treatment at Disease Remission. Disease remission occurred at day 23 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for Normal mice (n=3), and those in the RR-EAE groups receiving either saline (n=3) or LDN (n=5). Symbols represent averages for each smear. Significantly different from Normal at P<0.05 (*), P<0.01 (*), or P<0.001 (**). A) The RR-EAE+LDN mice showed significance from the Normal (***) and RR-EAE+Saline (**). D) RR-EAE+LDN showed significance from the Normal (*). E) The RR-EAE + Saline mice had a mean score of 3.3± 0.2 and the RR-EAE + LDN mice had a mean score of 2.5 ± 0.4.
Figure 4-3 Differential Cell Count and Average Behavioral Score for RR-EAE with LDN Treatment at Disease Relapse. Disease relapse occurred at day 32 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for Normal mice (n=5), and those in the RR-EAE groups receiving either saline (n=1) or LDN (n=2). Symbols represent averages for each smear. Significantly different from normal P<0.01 (**), or P<0.001 (**). (E) The RR-EAE + Saline mice has a mean clinical behavioral score of 3.0 ± 0.0 and the RR-EAE + LDN mice had a mean score of 3.0 ± 0.
Chapter 5: CH-EAE with OGF Treatment

5.1 Study Structure and Hypothesis

Although more than 85% of patients with MS present with RR-MS, most progress to a chronic, progressive form after a few decades. The mouse model for CH-EAE utilizing MOG$_{35-55}$ immunization has been shown to respond to OGF treatment with decreased clinical behavior and reduced neuropathology (Rahn et al., 2011; Campbell et al., 2012). For comparison to the RR-EAE model, we established mice with CH-EAE and evaluated blood samples at disease onset and peak disease. In addition to mice with CH-EAE, we examined leukocyte number and distribution in a group of animals treated with OGF. These data would allow comparisons of leukocyte distribution between two different animal models of autoimmune disease.

The CH-EAE model is characterized by disease onset and peak disease followed by a constant state of disease, unlike the RR-EAE model, which has distinct remissions and relapses over the course of the disease model. This model is used to represent the progressive forms of MS, primarily PPMS, which accounts for approximately 10% of all MS patients (NMSS, 2104). Previous studies on EAE and OGF have shown that treatment of EAE with OGF will result in amelioration of disease (Campbell et al., 2012; Hammer et al., 2013). In the CH-EAE model and in the RR-EAE model, the mice treated with OGF showed a reduction in disease severity over the control mice, as seen in a reduction in the behavioral scores in the mice treated with OGF. The hypothesis for the proposed study is that there will be differences in leukocyte numbers between the CH-
EAE and non-immunized control mice, and that these differences may correlate with disease progression. In addition, we hypothesize that leukocyte distribution differs in CH-EAE mice treated with saline or treated with OGF.

For this study, mice with CH-EAE were randomized at disease onset to receive daily injections of either saline or OGF. Behavior was observed daily. Blood was collected at baseline, disease onset, peak disease, and disease termination.

5.2 Behavior

Unlike the RR-EAE model, mice with CH-EAE do not display relapses and remissions. CH-EAE mice demonstrate disease onset, peak disease, and then disease scores plateau for the duration of observation. Non-immunized control mice display no adverse clinical behavior.

Disease onset for the CH-EAE infected mice occurred on day 10 post induction of MOG35-55. This point of the disease progression, the CH-EAE mice had an average behavioral score of 1.6 ± 0.1. Following this point in the disease progression, the CH-EAE mice were randomly assigned to receive daily injections of saline or 10 mg/kg OGF.

Peak disease for the CH-EAE mice occurred on day 19 post induction of MOG35-55. The CH-EAE mice treated with saline had a mean behavioral score of 6.2 ± 0.2. The CH-EAE mice had a mean behavioral score of 3.0 ± 0.0. This is a significant reduction in the disease score over the saline treated CH-EAE mice.
Mice were euthanized on day 27 after MOG\textsubscript{35-55} immunization. At this point the CH-EAE mice treated with saline had a mean behavioral score of 5.1 ± 0.2, and mice receiving OGF had a mean behavioral score of 6.8 ± 0.3.

5.3 Total Leukocytes

Total leukocyte values at baseline and disease onset for mice in the RR-EAE or CH-EAE models are presented in Figure 3.1 and are comparable. The average baseline leukocyte level for the RR-EAE model was 56.8 ± 4.7, and the average baseline leukocyte level for the CH-EAE model was 60.7± 8.9.

Similar to the RR-EAE model, the mice with CH-EAE had significantly more total leukocytes than non-immunized control mice (Figure 5.1A). The non-disease control mice had a mean of 55.8 ± 2.1 leukocytes, while the CH-EAE mice had a mean of 339.1 ± 14.2, an increase of 508%; total leukocytes populations differed at P<0.0001.

Figure 5.2A shows the total leukocyte levels at peak disease. The non-immunized control mice had a mean total leukocyte number of 70 ± 3.5 in comparison to 86.3 ± 12.0 for mice in the CH-EAE treated saline group. CH-EAE animals receiving OGF had a mean total leukocyte value of 149.0 ± 9.6. OGF significantly increased (P<0.001) leukocyte number by more than 73%. The posttest revealed that the differences between the non-diseased control mice, and the CH-EAE mice treated with OGF, as well as between the two CH-EAE treatment groups.

Figure 5.3A shows the total leukocyte levels at the time of euthanization. The non-immunized control mice averaged 46.5 ± 7.0 leukocytes in comparison to CH-EAE mice treated with saline that had 30.0 ± 4.5 leukocytes and CH-EAE mice treated with
OGF that had a mean total leukocyte number of 38.5 ± 8.0. No differences were noted in the CH-EAE groups.

5.4 Differential Leukocytes

Figure 5.1 B shows the neutrophil percentages at disease onset. The non-immunized control mice had a mean percent neutrophil of 11.3% ± 1.9% in comparison to 25.1% ± 2.9% for CH-EAE animals, a 2-fold increase over the normal controls. The difference between the groups is significant (P=0.04).

Figure 5.1C shows the eosinophil percentages at disease onset. Normal mice had a 4.1% ± 1.0% distribution of eosinophils, in comparison to CH-EAE mice that had 0.9% ± 0.1% esoinophils in a population of 200 leukocytes; this difference is significant at P<0.001.

Lymphocyte percentages at disease onset are shown in Figure 5.1D. The non-diseased control mice had a mean percent lymphocyte distribution of 64.6% ± 17.5%, and the CH-EAE mice had a lymphocyte distribution of 60.3% ± 3.5%. These values did not differ. The percent monocytes within 200 leukocytes did not exceed 0.7% of the total leukocytes for both non-disease controls and CH-EAE mice (0.51% and 0.069% respectively). The differences between these groups were not significant.

Peak disease occurred on day 19 post MOG35-55 induction. Figure 5.2B shows the percent neutrophils at peak disease. The non-immunized control mice had a mean percent neutrophil of 4.7% ± 0.7%. The CH-EAE mice treated with saline had a mean percent neutrophil of 20.3% ± 4.2%, more than a 4-fold increase. However, CH-EAE mice treated with OGF had a mean percent neutrophil of 37.9% ± 5.6% which represented a 3-
fold increase over saline treated EAE mice, and nearly 10-fold more cells than in normal controls. The differences between the groups was shown to be significant (P=0.0093), where the CH-EAE mice treated with OGF had significant differences from the non-diseased control mice.

Figure 5.2C shows the eosinophil percentages at peak disease. The non-immunized control mice had a mean percent eosinophil of 1.0% ± 0.4%. The CH-EAE mice treated with saline had a mean percent eosinophil of 1.7% ± 0.3%, an increase of 63%. The CH-EAE mice treated with OGF had approximately 1.0% eosinophils from the total leukocytes, which was similar to the control mice, and a reduced value to those receiving saline. These differences were not significant. Behavior at the time of sampling these bloods is presented in Figure 5.2E.

Percent lymphocytes for the peak disease collection point are shown in Figure 5.2D. The means for the three groups were similar, with means of 104.9% ± 7.6%, 76.0% ± 6.3%, and 48.0% ± 7.4% for the control, saline treated mice, and mice treated with OGF, respectively. These differences were shown to be significant (P=0.0004), where the CH-EAE mice treated with OGF had significant differences from the CH-EAE mice treated with saline and the non-diseased control mice. Percent monocytes for all three treatment groups did not exceed 0.4% (0.0% for the non-disease control, 0.4% for the saline treated CH-EAE mice, and 0.0% for the LDN treated mice). The differences between these groups was shown to be significant (P=0.0032). The posttest revealed that there were significant differences between the non-diseased control mice and the CH-EAE mice treated with OGF, as well as between the CH-EAE treatment groups.
Figure 5.3B shows the neutrophil percentages at disease termination. The non-diseased control mice had a mean percent neutrophil of 12.5% ± 4.0%. The CH-EAE mice treated with saline had a mean percent neutrophil of 25.9% ± 3.2%. This is an increase of almost 42% over the non-disease control. The CH-EAE mice treated with OGF had a mean percent neutrophil of 19.8% ± 6.2%. This is an increase of almost 13% over the non-disease control and a decrease of almost 21% over the CH-EAE mice treated with saline. An ANOVA was conducted and revealed a P-value of P=0.2189 suggesting that the differences between the groups were not significant.

Figure 5.3C shows the eosinophil percentages for disease termination. The non-disease control mice had a mean percent eosinophil of 1.1% ± 0.2%. The CH-EAE mice treated with saline had a mean percent eosinophil of 3.7% ± 1.2%. This is an increase of over three-fold from the non-disease control. The CH-EAE mice treated with OGF had a mean percent eosinophil of 1.7% ± 1.1%. This a decrease of 20% over the non-disease increase mice and a decrease of 50% over the CH-EAE mice treated with saline. The differences between the groups were found to be not significant.

Lymphocyte percentages for disease termination are shown in Figure 5.3D. The non-diseased control had a mean lymphocyte percent of 46.7% ± 3.7%. The CH-EAE mice, both saline-treated and OGF-injected mice had similar percentages, with means of 49.7% ± 7.8% and 46.8% ± 7.8, respectively. There was no significance to the differences between the groups. The percent monocytes were similar for the non-diseased control mice and the CH-EAE mice treated with saline, each with a mean of 0.0%. The CH-EAE mice treated with OGF had an increased mean percent monocyte (0.5%), however these differences were found to be not significant.
Figure 5-1 Differential Cell Count and Average Behavioral Score for CH-EAE Mice with OGF Treatment at Disease Onset. Disease onset occurred on day 9 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), and lymphocytes (D) for Normal mice (n=2), and those in the CH-EAE group (n=5). Symbols represent averages for each smear. Significantly different from Normal at P<0.05 (*) and P<0.001 (***). E) The average behavioral score for the RR-EAE mice at this point was 1.4 ± 0.1.
Peak Disease

Figure 5-2 Differential Cell Count and Average Behavioral Score for CH-EAE with OGF Treatment at Peak Disease. Peak disease occurred at day 19 post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), eosinophils (C), lymphocytes (D) for Normal mice (n=1), and those in the CH-EAE groups receiving either saline (n=3) or OGF (n=3). Symbols represent averages for each smear. Significantly different from Normal at P<0.05 (*) and at P<0.01 (**). A) The CH-EAE+OGF showed significance over both groups of CH-EAE+Saline and Normal mice (**). B) The CH-EAE+OGF showed significance over the normal (*). D) The CH-EAE+OGF showed significance over the CH-EAE+Saline (*) and Normal (**) groups. (E) The CH-EAE + Saline group has a mean score of 6.2 ± 0.2 and the CH-EAE + LDN mice had a mean score of 3.0 ± 0.0.
Figure 5-3 Differential Cell Count and Average Behavioral Score for CH-EAE with OGF Treatment at Disease Termination. Mice were terminated 27 days post disease induction. Blood smears were analyzed for total leukocytes (A), neutrophils (B), and eosinophils (C), and lymphocytes (D) for Normal mice (n=1), and those in the CH-EAE groups receiving either saline (n=6) or OGF (n=3). Symbols represent the averages for each smear. The CH-EAE + Saline mice has a mean behavioral score at the time of euthanasia of 5.1 ± 0.2 and the CH-EAE + LDN mice had a mean score of 6.8 ± 0.3.
Chapter 6: OGF – OGFr Biomarker

The blood collected throughout the course of these studies was centrifuged, and serum frozen for future analyses. Specifically, three different ELISAs were proposed: mouse-specific Met-Enkephalin (MyBioSource), mouse-specific Met-Enkephalin receptor or OGFr, and Serum Amyloid P, an indicator of acute inflammatory protein, Serum Amyloid P (Pepys et al 1979).

Several limitations prevented conducting the assays. The primary concern related to the encephalin ELISA was over the sensitivity of the assay and the concentration of Met-Enk in the blood samples. The assay detected levels higher than the than the concentration of the Met-Enk in the blood samples. Regarding the Met-Enk receptor assay, there was no published reports of OGFr levels in the blood. Preliminary ELISAs would have been required to collect data for both the Met-Enk and its receptor. At this point, the ELISAs were not completed and deemed too costly. However, it was expected that the OGF treated mice, and the LDN treated mice would show higher levels of Met-Enk and higher levels of the Met-Enk receptor than would either the non-disease control mice or the EAE mice treated with saline.

The SAP ELISA was limited by low amounts of blood being taken per collection point. In order to have adequate amounts of serum to conduct the ELISA, the serum samples from all the mice in a group would have to be combined, and this would reduce the accuracy of the assay. However it may be expected that the SAP levels would increase at disease onset and at peak disease. At peak disease however, the mice
receiving LDN or OGF treatment would see a reduction from those receiving saline treatment. For the RR-EAE model, there would be a corresponding increase and decrease in the SAP levels with a relapse and remission, respectively. It might be expected that the RR-EAE mice treated with LDN would have a reduction in the SAP levels over the saline treated RR-EAE mice at the same point in the disease progression.

In addition a western blot was conducted on the serum samples to compare the serum levels of the chemokine CXCL16. Chemokine levels, such as CXCL16, have been shown to increase with MS flares and decreases with treatment of MS (Graber and Dhib-Jalbut, 2011). CXCL16 has been shown to correlate with changes in RRMS severity (Holmoy et al., 2013). CXCL16 has also been associated with EAE, and shown to be expressed in spinal cords of mice with EAE (Szczuczynski, 2007). While the antibody for CXCL16 was not available for this serum electrophoresis, the blot was stained with Ponceau stain. This stain displayed the banding pattern of the serum samples quelling issues of protein degradation due to the age of samples and freezer failure.
Chapter 7: Histopathology

Spinal cords from RR-EAE mice treated with saline or LDN were collected for histopathology. Tissues were processed for histological staining of mast cells and activated astrocytes. Images of spinal cord tissue from RR-EAE mice receiving saline are shown in Figures 7.1 and 7.2. Increases in mast cells (Figure 7.1) and activated astrocytes (Figure 7.2) are noted in EAE mice relative to Normal animals (data not shown).

The spinal cords were collected at the termination of the study, when the mice were in a period of remission. An ANOVA was conducted for the mast cell counts and activated astrocyte counts and revealed significant differences between the non-disease control mice and the RR-EAE mice. However, there were no differences between the RR-EAE mice treated with saline and the RR-EAE mice treated with LDN for both the mast cell and the activated astrocytes.

Tissue from mice with CH-EAE and receiving OGF were not processed, as their behavioral scores did not indicate responsiveness to the therapy.
Figure 7-2 Toluidine Blue Staining of Spinal Cord of RR-EAE Mice. Photomicrograph of spinal cord from RR-EAE mouse at time of disease remission. Note an increased in number of inflammatory mast cells. Magnification 200X

Figure 7-1 GFAP Staining of Spinal Cord of RR-EAE Mice. Photomicrograph of spinal cord from RR-EAE mouse at time of disease remission. Note the elevated level of activated astrocytes within the white matter in the RR-EAE mouse. Magnification 200X
Chapter 8: Discussion

These studies have shown that evaluation of peripheral blood to assess the numbers of total leukocytes, neutrophils, and eosinophils provided relevant markers for progression of both RR-EAE and CH-EAE. Differential cell counts also were modified following OGF or LDN therapy, particularly at peak disease.

Comparison of the two strains of mice revealed that baseline levels of leukocytes were comparable between the different strains suggesting that analysis of peripheral blood for markers of disease progression could be conducted for both EAE animal models. Moreover, even though the disease model of EAE was markedly different between SJL/CRL and C57Bl6/J mice, the two different models could be compared for their shared points along disease progression, namely disease onset and peak disease.

Disease onset shows the most significant difference within the models. At disease onset for the RR-EAE model and the CH-EAE model there was a large increase over the control mice. Interestingly, the increase for the CH-EAE model at disease onset was larger than the increase for the RR-EAE mice. This is likely because the CH-EAE model requires two injections of the CFA, one with the MOG_{35-55} at day zero, and one without at day seven. The RR-EAE model only requires the injection of CFA at day zero with the PLP_{139-151}.

During the course of RR-EAE, evaluation of peripheral blood demonstrated that mice with disease had significantly higher levels of leukocytes, neutrophils, and
eosinophils, in comparison to normals. As expected, differential cell counts for non-immunized mice did not change over the study period.

Induction of RR-EAE resulted in an elevation in leukocyte number as well as neutrophils; these values became reduced as the disease progressed through remission and relapse. The number of eosinophils ranged between 1 and 3 in non-immunized mice, and ranged between 2 and 5 in RR-EAE animals; despite the small overall numbers, this represented 2-fold increases in eosinophils.

Treatment of RR-EAE mice with LDN has been shown to markedly reduce clinical disease. However, in this study, the mice did not respond robustly to LDN. Studies have suggested that this biphasic effect may be a result of the mouse strain or model of induction (DeLuca et al., 2010). However, RR-EAE mice receiving LDN did demonstrate reductions in overall leukocyte number, and differential counts of neutrophils and eosinophils, at peak disease (Fig. 8.1). During remission, RR-EAE + LDN animals had differential cell counts comparable to normal mice. There was a sharp increase in the eosinophil levels for the RR-EAE + LDN mice at disease relapse, which is indicative of inflammation, and a return to a diseased state.

Evaluation of the progressive model of EAE that utilizes MOG_{35-55} immunization revealed marked elevation in total leukocytes, neutrophils, and lymphocytes at disease onset. The increase in total leukocytes and neutrophils remains through peak disease (Figure 8.2). The number of eosinophils in the CH-EAE mice does not fluctuate over the course of EAE from that of normal C57Bl6/J mice.
OGF treatment of CH-EAE animals did not permanently diminish behavioral symptoms in this study. There was a decrease in behavioral symptoms at peak disease; however this decrease in behavioral symptoms was reversed by the end of the study. Differential blood cell counts revealed significant elevation in leukocytes and neutrophils in OGF-treated mice relative to CH-EAE mice. The mechanism for these differences, as well as determination of their validity, requires further study.

Previous research has shown that treatment of T and B lymphocytes with endogenous OGF has an inhibitory effect on the proliferation of these cells (Zagon et al., 2011; Zagon et al., 2011). In a similar fashion, modulation of the OGF-OGFr axis appears to reduce the proliferation of the leukocytes in the peripheral blood, as seen with the treatment of RR-EAE with LDN. This effect was seen most predominantly at peak disease, where there was a significant reduction in both total leukocyte levels and neutrophil levels for the mice treated with LDN. In addition to this reduction at peak disease, mice treated with LDN had a reduction in peripheral lymphocytes at disease remission.

While the strongest results came from disease onset and peak disease, these studies have shown that there are measurable differences in the peripheral blood that change with the disease progression. The blood cell measurements made in these studies are part of routine diagnostic measurements that can be conducted in a primary care setting. However blood cells alone will not give a complete description of the disease, since an immune response similar to what was seen in these studies is a hallmark of inflammatory diseases. Another biomarker needs to be measured in conjunction with the cell counts to full track the disease progression of MS.
This study is not without its limitations and issues that must be discussed. First and foremost, the EAE model of MS is not exactly like MS. In addition, mice are not exact analogues of humans. The leukocyte distribution of the mouse is not the same as the human. Mice are decidedly lymphphilic while humans are mainly neutrophilic (Ross and Pawlina, 2011; Jackson Laboratories, 2007; Jackson Laboratories, 2012). Another major difference between EAE and MS is that EAE is an induced disease while MS is a spontaneous disease (Constantinescu et al., 2011). In addition, the lack of a robust response, lack of severe disease in the RR-EAE mice treated with saline, and the reversal in the disease severity in the CH-EAE mice treated with OGF all show issues that work to compromise the integrity and the validity of the study.

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The blood smears also were a point show limitations of the study. Even when prepared perfectly, the smears, at best, are only a representative section of the total blood cell distribution. This representative section is not as accurate as other more clinically oriented blood tests, such as a CBC, which has become a staple of clinical blood tests. These more accurate tests would provide better insight and a more accurate description of the leukocyte distribution in the mice.

The wide spread of the percent differential leukocytes as well as the lack of significant and robust response to the EAE displayed another issue with the study. The
overlapping values seen in the blood smear data demonstrates that the data is semi-quantitative at best, and inaccurate. All of the significances that were found between the groups display a statistical significance; however this does not show a clinical difference. While the statistical significance can demonstrate differences between the groups, it is the spread of values within a group that raises concerns over the clinical significance. The wide range of values, even with the non-diseased control mice indicates not only possible variation in the mice themselves, but also the inaccuracies of the blood smears. One way to possibly counter the variability seen in the groups and remove some of the outliers in the groups would be to increase the number of mice in each treatment group. In theory, more mice per treatment group would give a tighter distribution of values and give a value closer to the actual mean. Also a more accurate method of leukocyte analysis, such as a CBC, would help to reduce the variability seen in distributions of cells.

In addition to the above mentioned issues, the method in which these mice where housed could account for some variability in the distribution of leukocytes, even within the treatment groups. The mice were housed in traditional open-top cages and were isolated from other mice in the facility. While in theory, the isolation of the EAE mice should have shielded them from many outside pathogens and opportunistic infections; it is still possible that the mice could be infected with some outside pathogen. In order to prevent infection from outside infections the mice should be kept in isolated cages designed for immunocompromized mice, where each cage is receiving its own filtered air supply.

Another limitation of the study came from when the blood was taken. The intent of the experiment was to be able to track the biomarkers in the blood from individual
mice over the course of the disease. However, due to the amount of blood that was needed for the smears and the planned ELISAs, the mice could only have blood drawn once every two weeks. This limited survival collections though the submandibular vein to every other collection point for a mouse. In this respect, studying blood biomarkers would be more effective in MS, rather than in the EAE model. Blood samples taken from humans would yield sufficient serum for several analyses. In addition, the first collection point after the pre-immunization baseline was at the onset of clinical disease, which is after the neurological damage, has already begun to occur. This indicates that some of the changes that were expected to be seen likely occurred more before the onset of clinical symptoms. The study of the preclinical, pre-onset biomarker changes in the peripheral blood is both beneficial to MS diagnosis and is also not totally practical. It is very important to understanding what changes are occurring in the patient prior to the onset of clinical disease in order to determine a set of diagnostics for the disease. On the converse of that, most patients do not go to a physician with concerns of a disease prior to the onset of clinical symptoms. In that aspect, it is more important to understand the changes that occur from the onset of symptoms, to the peak of a flare, and to back to a remission.

There is the potential for one more treatment group in this study. While the RR-EAE and CH-EAE mice treated with saline acted as a positive control, the study would have benefited from a negative control group. This negative control group would come in the form of EAE mice treated with the opioid antagonist Naloxone. While the Naloxone would not be expected to ameliorate the EAE model, it would remove the effects of any of the endogenous opioids in the mice, including the effects of OGF, and therefore could potentially have an effect on the distribution of leukocytes.
Finally, and perhaps the largest limitation of this study is the lack of specificity in that comes with analysis of leukocyte levels. The immune response and changes in immune cell levels is not exclusive to EAE or MS. There are a number of diseases, conditions, and infections that will cause a change in the immune cell levels. Without finding a biomarker that is unique to EAE and to MS, a diagnostic test with peripheral leukocyte data alone would only signify the presence of disease, and nothing more.

A recent study, blood samples from patients with RRMS, PPMS, and SPMS were compared to determine what differences exist between the types of MS (Dickens et al., 2014). The aim was to find what biomarker, if any, could signal the transition from RRMS to SPMS. This study found some differences in metabolites and fatty acids between a healthy control and MS patient, and between RRMS and SPMS patients, but not between PPMS and SPMS. However these findings were compared to other neurodegenerative disorders and the metabolite levels were found to be disease specific. In addition, these metabolite levels were found to be not affected by MS medications. This means that these metabolite levels can potentially be used for diagnostic purposes.

Another recent study formed a panel of proteins found in the serum of MS patients that can be used as to classify the severity of MS, in particular RRMS (Tremlett et al 2015). In this study, a series of eleven proteins, including: Thrombospondin-1, Cholinesterase, Platelet glycoprotein V, and Lipopolysaccharide-binding protein. This panel of proteins demonstrated differences between aggressive disease progression and benign disease progression, and presents a possible diagnosis panel for peripheral blood.
These investigations may provide some insight into the status of MS patients. While the metabolite study and the proteomic study hold the promise of a primary diagnostic tool, the blood cell biomarker studies would be more akin to tracking the course of disease. The need to assess the progression of the disease, and ultimately predict when a disease flare will occur is important for treatment of MS patients. If predictions can be made as to when a flare will occur, a proactive treatment for the flare can be implemented, rather than starting a reactive treatment after the flare has begun. Thus, preventive measures to reduce the number or severity of flares would improve the quality of life for MS patients.

The most important aspect of these tests is the convenience and the simplicity of the tests for the patients. Currently, the methods to assess and diagnose MS include MRIs and analysis of CSF (Polman et al., 2011). These procedures are not a normal part of the primary care physician’s repertoire, while collecting blood samples to monitor things, such as cholesterol, is a part. Since it has now been shown that there are different biomarkers that can be tracked in the peripheral blood of MS and its model, EAE, it is reasonable to continue research for additional peripheral blood biomarkers that will enable assessment of disease progression, as well as response to therapy, for MS.
Figure 8-1 Summary of Cell Levels for the RR-EAE Model Across Disease Progression. Diagram representing total leukocyte, neutrophil, eosinophil, and lymphocyte levels in the RR-EAE mouse model over the course of disease progression.
Figure 8-2 Summary of Cell Levels for the CH-EAE Model Across Disease Progression. Diagram representing total leukocyte, neutrophil, eosinophil, and lymphocyte levels in the CH-EAE mouse model over the course of disease progression.
References


