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The Role of MERTK in Central Inflammatory Demyelination

Dr Christopher Michael Dwyer

ORCID ID 0000-0001-6387-6585

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The Florey Institute of Neuroscience and Mental Health

Department of Anatomy and Neuroscience

The University of Melbourne

*Dedicated to my father,
with love and admiration*

ABSTRACT

Multiple sclerosis (MS) is, at least in part, an autoimmune demyelinating disease of the central nervous system. It is the most common cause of neurological dysfunction in young adults. In addition to demyelination, autoimmune destruction in MS results in oligodendrocyte loss, local inflammation and neurodegeneration. Later in the disease course, a pattern of neurodegeneration may emerge without obvious evidence of adaptive immune activation. Current evidence implicates dysregulation of the innate immune system as a major contributor to the neurodegeneration of progressive MS. It is well established that innate immunity influences central nervous system damage and modulates myelin repair.

The TAMs (TYRO3, AXL and MERTK) are a family of receptor tyrosine kinases expressed by discrete innate immune cell types, including macrophages, microglia and dendritic cells. They are involved the homeostatic regulation of adult, fully differentiated tissues that are subject to constant intrinsic and environmental challenge. The primary aim of this thesis was to provide additional insight into the biology of the MERTK receptor by examining its role in commonly employed mouse models of MS. In addition, I sought to identify whether serum levels of MERTK correlated with disease activity in MS patients experiencing relapse.

I established that heterozygote deletion of *Mertk* in CD11^{+ve} cells worsens the clinical severity of EAE in male mice, demonstrating for the first time that changes in the expression of a TAM receptor can alter the outcomes of a model of MS. Conversely, homozygous deletion did not result in EAE exacerbation. Heterozygote deletion of *Mertk* in CD11^{+ve} cells does not alter the outcomes of cuprizone-induced demyelination, suggesting that CNS-resident CD11c^{+ve} cells (microglia) are not implicated in the clinical exacerbation observed in EAE cohorts. In human subjects, MERTK expression by monocytes and dendritic cells does not correlate with periods of increased inflammatory activity in MS. MERTK expression by circulating dendritic cells is very limited.

Taken together, the data in this thesis offer compelling evidence that MERTK is an important regulator of the outcomes of central inflammatory demyelination. They justify further research efforts in this area to better understand the mechanism of these disease states and to develop therapeutic targets for the treatment of MS.

DECLARATION

I certify that:

1. The thesis comprises only my original work towards the Doctor of Philosophy, except where indicated in the preface.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Dr Christopher Michael Dwyer

MS Division

Florey Institute of Neuroscience and Mental Health

Department of Anatomy and Neuroscience

The University of Melbourne

Parkville, AUSTRALIA

PREFACE

The experimental data presented in this thesis comprises only my original work, except for the following results, obtained in collaboration with others:

1. Data regarding TAM receptor and ligand expression in health and disease were originally generated by Dr. Gerry Ma.
2. Experimental Autoimmune Encephalitis (EAE) experiments and immunohistochemical analysis in WT animals were performed by Michele Binder and Dr. Gerry Ma.
3. All EAE inductions were performed with the assistance of Michele Binder, Andrea Aprico, Farrah Blades or Linda Nguyen.
4. Microsatellite analysis of *Tyro3* alleles was performed by the Australian Genome Research Facility.
5. The preparation of human histochemical samples from patients with secondary progressive multiple sclerosis was performed by Ranjan Dutta and Ajai Tripathi at Cleveland Clinic, Lerner College of Medicine, Case Western Reserve University, Cleveland, OH.

Accordingly, I assess my total contribution to the experimental data presented in this thesis as follows:

Chapter 3: 50%

Chapter 4: 95%

Chapter 5: 80%

Chapter 6: 95%

The publication status of the results chapters of this these are set out below:

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Trevor and Michele are also responsible for fostering a culture of respect and support in the broader MS Division. I benefited greatly from the experience and guidance of the honours and PhD students within the group, fellow travellers on this journey. They offered a wealth of practical tips and tricks that greatly assisted me when roadblocks stood in my way. In particular, I would like to thank Farrah Blades for her guidance with relation to the many EAE experiments set out in this thesis. Alex Walsh provided expert advice with regard to molecular techniques and Linda Nguyen introduced me to the joys of cuprizone intoxication. Last but by no means least, Dr. David Gonzalez was instrumental in demonstrating a rapid technique for spinal cord dissection and lumbar expansion isolation. Without his assistance, I would still be in the lab removing spinal lamina!

I was also fortunate to benefit from the experience and guidance of expert research assistants. Andrea Aprico, Laura Johnson and Eze Nwoke approached my questions and entreaties for help with patience and kindness and the successes I enjoyed in the lab are built on their expertise. I would also like to note the special contribution of Dr. Vanta Jameson who assisted with nearly all of the flow cytometry experiments presented in this thesis and was instrumental in troubleshooting the many technical challenges I encountered.

My greatest appreciation is owed to my partner, Leesha and my beautiful children, Marcus and Sophia. I was absent for key moments in their lives in furtherance of the goals of this thesis. Their love, support and forbearance are imprinted on the pages that follow.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance
APC: antigen presenting cell
BAC: bacterial artificial chromosome
BBB: blood-brain barrier
BMDM: bone marrow-derived macrophage
BMDC: bone marrow-derived dendritic cell
CD: cluster of differentiation
cDNA: complementary deoxyribonucleic acid
CI: confidence interval
CIS: clinically isolated syndrome
CNS: central nervous system
CSF: cerebrospinal fluid
Cuprizone: bis-cyclohexanone oxaldihydrazone
Cxcr4: chemokine (C-X-C motif) receptor 4
DC: dendritic cell
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
dNTPs: deoxyribonucleoside triphosphates
DPBS: Dulbecco's phosphate buffered saline
EAE: experimental autoimmune encephalomyelitis
EDSS: Expanded Disability Status Scale
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
ES: embryonic stem
FACS: fluorescent-activated cell sorting
FC: fragment crystallisable
FCS: foetal calf serum
fl: floxed

FNIII: fibronectin type III
GAS6: growth arrest-specific gene 6
Gla: γ -carboxyglutamic acid-rich
GTPase: guanosine-5'-triphosphatase
GWAS: genome-wide association study
HC: healthy control
HLA: human leukocyte antigen
HRP: horseradish peroxidase
HSP: heat shock protein
IBA1: ionised calcium-binding adapter molecule 1
ICAM: intracellular adhesion molecule
IFN: interferon
Ig: immunoglobulin
IL: interleukin
i.p.: intraperitoneal
LD: linkage disequilibrium
LN: lymph node
MAC3: Microglia/macrophage activation marker
MACS: magnetic-activated cell sorting
MBP: myelin basic protein
MHC: major histocompatibility complex
MHC-I: MHCII: major histocompatibility complex class I
MHC-II: major histocompatibility complex class II
MIM: Online Mendelian Inheritance in Man code
MOG: myelin oligodendrocyte glycoprotein
MS: multiple sclerosis
MT-PBS: mouse tonicity phosphate buffered saline
NF κ B: nuclear factor- κ B
NOD: nonobese diabetic
OCB: oligoclonal band

OCT: optimal cutting temperature
OPC: oligodendrocyte precursor cell
PCR: polymerase chain reaction
PDGFR α : α -type platelet-derived growth factor receptor
PEG: polyethylene glycol
PFA: paraformaldehyde
PLP: proteolipid protein
PPMS: primary-progressive multiple sclerosis
ProS: Protein S
RNA: ribonucleic acid
RNase: ribonuclease
RPE: retinal pigment epithelial
RRMS: relapsing-remitting multiple sclerosis
RT: reverse transcriptase
RTK: receptor tyrosine kinase
SD: standard deviation
sEAE: spontaneous EAE
SEM: standard error of the mean
SLE: systemic lupus erythematosus
SNP: single nucleotide polymorphism
SPMS: secondary-progressive multiple sclerosis
STAT: signal transducer and activator of transcription
TAE: Tris-acetate-EDTA
TAM: Tyro3, Axl, Merck
TK: tyrosine kinase
TLR: Toll-like receptor
TNF: tumour necrosis factor
Treg: regulatory T cell
TH: helper T cell
WT/wt: wild-type

LIST OF MEASUREMENTS

dpi: days post induction

°C: degree Celsius

g: gravitational force

g: gram

kg: kilogram

μM: micromolar

μm: micrometre

μl: microliter

μg: microgram

M: molar

min: minute

mg: milligram

mM: millimolar

ml: millilitre

mm: millimetre

nM: nanomolar

ng: nanogram

nm: nanometre

%: percentage

rpm: rotations per minute

U: units of activity

v: volume

w: weight

CHAPTER 1: INTRODUCTION

1.1 Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system. It is the most common cause of neurological dysfunction in young adults. In addition to demyelination, autoimmune destruction in MS results in oligodendrocyte loss, local inflammation and neurodegeneration.

The pathophysiology of MS is complex and involves interactions between local neural, glial and immune cells, along with infiltrating peripheral leukocytes. Emerging evidence implicates the TAM (TYRO3, AXL and MERTK) family of tyrosine kinases in the modulation of central nervous system (CNS) responses to tissue injury and autoimmune inflammation. In this chapter, MS and its experimental models will be reviewed along with central responses to immune-mediated demyelination and the role of TAM signalling in autoimmune disease.

1.2 Multiple Sclerosis

Multiple sclerosis (MIM 126200) is a chronic autoimmune disease that causes progressive demyelination and destruction of the CNS. Although the disease is highly heterogeneous between individuals, established patterns of clinical, radiological and histopathological change allow for the description of a common disease entity.¹ Autoimmune demyelination results in plaque formation typically in the optic nerves, periventricular white matter, juxtacortical borders, cerebellum, brainstem and cervical spinal cord. In addition, patients also experience accelerated cortical atrophy.² These processes result in the accumulation of neurological deficits and disability. Symptoms experienced by patients may often be correlated with particular areas of lesion formation and include visual disturbance, ataxia, weakness, sensory changes, bowel and bladder dysfunction, fatigue and cognitive decline.³

Neurological deficits in MS can develop in either a step-wise or continuous manner. *Relapse* is defined as an acute or subacute deterioration in neurological function for a period of 24 hours or more, followed by partial or complete recovery. These events should occur without evidence of intercurrent fever or infection. *Progression* is defined as a continuous and gradual deterioration in neurological function without any discernible recovery in function.⁴

Approximately 90% of patients with MS initially experience a relapsing-remitting course of disease, with the first symptoms emerging in early adulthood. This is referred to as Relapsing-Remitting Multiple Sclerosis (RRMS). After a period of typically ten to fifteen years, most patients will experience a change in their disease. Episodes of relapse give way to the inexorable accumulation of neurological deficit. This is referred to as Secondary Progressive Multiple Sclerosis (SPMS). Approximately 10% of patients experience continuous symptom development from disease outset, without intermittent remission. This form of the disease is referred to as Primary Progressive Multiple Sclerosis (PPMS).⁵

Changes in disease phenotype reflect underlying differences in pathophysiology. While relapse episodes are associated with neuroinflammation, adaptive immune cell infiltration and oligodendrocyte loss,⁶ progressive phases of the disease reflect a process of neurodegeneration, with a greater proportion of innate immune cell activity.⁷⁻⁹ This change in pathophysiology is mirrored by varying clinical responses to disease modifying therapies. All approved MS therapies target the adaptive immune system and have resulted in improvements in clinical outcomes in RRMS. No disease-modifying agents specifically target innate immunity and until very recently, no treatments offered benefit for progressive forms of the disease.¹⁰

1.2.1 The Epidemiology and Risk Factors of Multiple Sclerosis

MS is the most common neurological disease of young adults. It affects approximately 21,000 individuals in Australia¹¹ and over 2.5 million worldwide.¹² The incidence of MS

is increasing, independent of greater disease awareness and improved diagnostic accuracy.¹² However, no cause for MS has been identified and the disease is thought to represent a complex interaction between genetic susceptibility and environmental factors. There may also be infectious or immune-stimulant factors that trigger the disease in individuals with the relevant genetic constitution and prior environmental exposures. Emerging evidence suggests that the gut microbiota is also altered in MS. A study in Japanese MS patients noted significantly less abundant *Clostridium* and *Bacteroides* species compared to healthy controls. Many of these species are capable of producing short-chain fatty acids that lead to expansion of the regulatory T cell (T_{reg}) population and maintenance of balance among species of gut microbiota appears to be crucial for immune homeostasis.¹³

While a detailed review of the susceptibility factors for MS lies outside the scope of this work, a number of key risk factors for the development of MS are briefly considered below.

1.2.1.1 Sex

Like many autoimmune diseases, MS is more common in women: the sex ratio is approximately 3.5:1.¹⁴ Genetic studies have not identified a risk gene on the X chromosome. Rather, the observed gender difference likely reflects changes in sex hormone levels. For example, paediatric MS demonstrates more evenly matched sex distribution than adult forms of the disease.¹⁵ In addition, pregnant patients with MS typically experience a period of disease quiescence during gestation when sex hormone levels are greatly increased. This is commonly followed by a surge in disease activity as those levels normalise in the puerperium.¹⁶ Indeed, progesterone and its derivatives have been shown to have anti-inflammatory and immunosuppressive effects¹⁷ in a sexually dimorphic manner.¹⁸ Their administration also reduces the severity of Experimental Autoimmune Encephalomyelitis (EAE), a commonly employed animal model of MS^{19, 20} and can induce anti-inflammatory immune cell polarisation.^{21, 22}

1.2.1.2 Genetic Associations

No single gene has been correlated with the risk of developing MS and genome-wide association studies have identified over 200 genes associated with increased MS risk.²³ The most prominent genetic association is with human leukocyte antigen (HLA) types in the major histocompatibility complex (MHC). However, HLA allele-associated risk is not distributed uniformly among populations. For example, the *HLA-DRB1*15* allele has long been associated with the development of MS in populations from northern Europe.²⁴ In contrast, population studies from Sardinia indicate a greater association with *HLA-DRB1*301*, *HLA-DRB1*0405* and *HLA-DRM1*1303*.²⁵ Other HLA alleles appear to confer protection against the development of MS.²⁶

1.2.1.3 Latitude

Studies from Australia and New Zealand demonstrate a strong risk association with greater extremes of latitude. The prevalence of MS in Hobart, Tasmania is 75 per 100,000 while in northern Queensland it is 11 per 100,000.²⁷ Similar findings are reported in New Zealand, where incidence is higher on the South Island compared to the North Island.²⁸ Although epidemiological studies from North America²⁹ and Western Europe³⁰ initially appeared to confirm a similar association, recent meta-analyses suggest an absence of latitudinal gradient in the Northern Hemisphere. In particular, when studies assessing incidence as opposed to prevalence are assessed, no latitudinal distribution is found in the Northern Hemisphere, although the Ausimmune incidence-based study did find such a gradient in the southern hemisphere.^{12, 31}

Clearly, ethnicity also contributes substantially to MS risk, regardless of latitude. The incidence of MS is far lower among the ethnic Sami of northern Norway than in neighbouring Caucasian populations.³² Similarly, rates of MS are considerably lower in the Maori population than among Caucasians in New Zealand.³³

1.2.1.4 Vitamin D

Sunlight exposure and thereby levels of vitamin D might account for the apparent latitudinal variation in MS rates reported in some studies. UVB radiation is required for the conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D₃) and past sunlight exposure has been found to be inversely correlated with MS development.³⁴ However, questionnaire studies about sunlight exposure are subject to substantial recall bias and results can be confounded by dietary sources of vitamin D. A study from Norway found no latitudinal change in MS incidence even though that country covers 24 degrees of latitude. Rather, a protective effect from the consumption of fatty fish and cod liver oil – both rich sources of vitamin D – was identified.³⁵ The relationship between sun-exposure, latitudinal gradient and vitamin D synthesis is obviously complicated in the context MS risk – data from the Ausimmune study found a protective effect from sun exposure following a first clinical demyelinating event but no correlation with serum 25(OH)D or vitamin D levels.³¹

1.2.2 The Pathophysiology of MS

Multiple sclerosis is characterised by the formation of demyelinating lesions or plaques throughout the central nervous system. These areas of focal damage demonstrate loss of myelin, oligodendrocyte death, astrocytic gliosis and the presence of infiltrating, activated immune cells of both the adaptive and innate immune systems.^{36, 37}

Oligodendrocytes are the glial cells of the CNS responsible for myelin production. Myelin provides not only protective insulation and trophic support for axons³⁸ but also facilitates saltatory conduction, greatly increasing the speed of action potential transmission.³⁹ Disability accumulates in MS through the loss of saltatory conduction and axonal degeneration, leading to a failure of neural transmission.⁴⁰ In addition to plaque formation, histological studies also demonstrate that a subacute process of demyelination and axonal loss can occur in normal appearing white matter (NAWM) and the cerebral cortex.³⁶

In response to immune-mediated demyelination, a process of remyelination is stimulated. In animal models, this is demonstrated by the presence of proliferating oligodendrocyte precursor cells (OPCs) in areas of central demyelination.^{41, 42} In addition, recent work has demonstrated that neural precursor cells (NPCs) residing in the subventricular zone have the potential to differentiate into oligodendrocytes in mouse models of central demyelination^{43, 44} as well as in human MS lesions.⁴⁵

However, despite the presence of myelinating oligodendrocytes, the degree of remyelination in human subjects is typically inadequate. Failure to sufficiently remyelinate damaged axons can be attributed to impaired progenitor cell recruitment and differentiation.^{46, 47} Very recent work has also demonstrated that only a minority of MS patients with very aggressive disease generate new oligodendrocytes in response to inflammatory demyelination. Carbon-14 dating in MS patients demonstrated that the oligodendrocytes in shadow plaques – areas of thinly myelinated white matter thought to represent areas of remyelination – were not newly generated from OPCs. This suggests that remyelination is predominantly performed by aged oligodendrocytes and may offer an avenue for therapeutic intervention by stimulating OPC maturation.⁴⁸

For many years, MS was considered a T cell mediated disease in which auto-reactive CD4⁺ T cells migrated to the CNS and caused local damage to nervous tissue. This view was largely informed by animal studies of EAE in which central demyelination could be elicited in disease-naïve animals by the injection of autoreactive myelin-specific CD4⁺ T cells.⁴⁹ However, alternative mechanisms are clearly involved in human manifestations of MS as clinical trials of monoclonal antibodies targeting CD4⁺ T cells did not improve disease outcomes.⁵⁰ In particular, seminal work by Barnett and Prineas demonstrated oligodendrocyte death in early MS lesions without any evidence of peripheral immune cell infiltration.³⁶ This indicates that in at least some lesions, oligodendrocyte loss and demyelination can be an exclusively local phenomenon.

The role of B cells in the development of MS has also recently been advanced by Jelcic and colleagues. A population of memory B cells that drive the proliferation of auto-reactive, brain-homing T-effector clones was found in MS patients during *remission* rather than relapse, as might have been presumed *a priori*. T-effector cell reactivity was induced in a HLA-DRB1*1501 positive MS patient by presentation of a peptide derivative of Ras Guanine Nucleotide Releasing Protein 2 (RASGRP2), a calcium sensor expressed within the CNS.⁵¹ Whether this pathogenic mechanism applies to the broader MS population and whether RASGRP2 is an initiator of disease or a reflection of epitope spreading awaits clarification. However, this important work at least partially accounts for the efficacy of B cell directed, anti-CD20 therapy in a disease typically thought to be T cell mediated. The presence of pro-inflammatory (Th1), CNS-targeting CD20⁺ve *T cells* in the blood and CSF of patients with MS, however, indicate that the mechanisms of adaptive-immune mediated demyelination require further elucidation.⁵²

As the majority of MS lesions include a diverse population of local and immune cells in various states of activation, it is now accepted that MS represents a complex network of central and peripheral immune-mediated destruction. This is evidenced by the fact that all approved therapeutics for the treatment of MS are immunomodulatory in their action.⁵³

1.3 Experimental Models of Multiple Sclerosis

A number of animal models of central demyelination have been developed in a bid to simulate the molecular and cellular changes that occur in MS. This effort is challenging as no other animal species appears to spontaneously develop autoimmune central demyelination and available models only address discrete aspects of the pathophysiology observed in humans.⁵⁴ This thesis employs the two most commonly used *in vivo* models of MS, EAE and cuprizone-induced central demyelination. Often, these two experimental systems are used in concert to examine different aspects of the human disease. EAE, in particular, is useful for examining immune responses that lead

to demyelination while cuprizone challenge simulates the results of oligodendrocyte loss.

1.3.1 Experimental Autoimmune Encephalomyelitis

The clinical phenotype manifested by EAE requires the induction of autoreactive CD4⁺ T cells raised against CNS myelin. This is achieved by inoculating study animals with an antigenic stimulant, typically myelin oligodendrocyte glycoprotein MOG₃₅₋₅₅ peptide (MOG), myelin basic protein (MBP) or proteolipid protein (PLP). Exposure to antigen alone, however, is insufficient to induce a consistent and reproducible immune response. Accordingly, the antigenic stimulant is first emulsified in Complete Freund's Adjuvant (CFA) which provides a suspension media from which antigen can elute over time. The addition of inactivated *Mycobacterium tuberculosis* (TB) to the suspension further insures the generation of a massive phagocytic response with subsequent antigen presentation. This in turn leads to the activation of CD4⁺ T cells and immune mediated destruction of tissue bearing antigenic signals commonly found in myelin. To further prime immune responses, the CFA/TB/antigen cocktail is supplemented by exposure to *Pertussis* toxin, typically on days 0 and 3 of EAE induction. The precise mechanism of the immune stimulus provided by *Pertussis* exposure is unknown, though theories involving the suppression of T cell anergy or T cell regulatory function, innate immune activation and increased blood-brain barrier (BBB) permeability have all been proposed.⁵⁵

Animals exposed to EAE develop an ascending paralysis that first involves the tail followed by the hind limbs. In more severe cases the forelimbs may also become involved. While the time course may vary, signs of motor weakness are typically evident by day 10 after induction with symptom peak between days 20 and 30. In C57BL/6 mice the disability accrued with this model is chronic, without obvious periods of significant remission.

The utility of the EAE model was demonstrated by its pivotal role in the development and commercialisation of multiple therapeutic agents for the treatment of MS.⁵⁶ However, it is subject to important limitations. In particular, EAE does not account for the heterogeneity seen in MS lesions where minimal inflammatory infiltration may occur despite marked demyelination and oligodendrocyte loss. Moreover, while EAE induces strong CD4⁺ T cell responses with subsequent axonal destruction and minimal demyelination, the majority of immune cells in human MS lesions are CD8⁺ T cells and macrophages where demyelination predominates.^{57, 58}

1.3.2 Cuprizone Challenge

Exposure to cuprizone (*bis*-cyclohexanone oxaldihydrazone), a copper chelator, results in oligodendrocyte loss and subsequent demyelination in experimental mouse models. This effect is more pronounced in particular regions of the brain, most notably the corpus callosum, hippocampus, cerebellum and caudate putamen.⁵⁹ It is not known why oligodendrocytes are preferentially affected or why focal lesions develop, although it has been posited that mitochondrial oxidative phosphorylation is disturbed.⁶⁰ Demyelination is induced by adding cuprizone 0.2% by weight to the standard mouse chow of C57BL/6 mice. Oligodendrocyte death can be observed after one week and by three weeks discrete areas of demyelination can be identified. Disease severity peaks after approximately five weeks. Concomitant with demyelination, profound levels of astrocyte and microglial activation are observed.⁶¹ Microglia, in particular, are implicated in the removal of myelin debris which is essential for eventual remyelination.^{62, 63}

Assessment of the degree of demyelination can be made with histological procedures (luxol-fast blue staining, LFB), immunohistochemistry (IHC) against myelin components, including MBP and PLP, electron microscopy, and gene/protein expression studies. With the withdrawal of cuprizone from animal feed, oligodendrocyte precursors develop and the process of remyelination can then be observed.

As with EAE, there are important caveats on the experimental observations made using the cuprizone model. In particular, as demyelination in this model is caused by direct toxicity there is no role for peripheral immune activation. Accordingly, the role played by T cells, B cells and macrophages in the development of MS are excluded from assessment.⁶⁴ This is an important limitation as all current approved therapeutics for the treatment of MS target peripheral immune cells.

1.4 Inflammatory Activity in Central Demyelination

As noted, one characteristic of MS lesions is the infiltration of inflammatory immune cells. Members of both the adaptive and innate immune systems are implicated in this process.⁶⁵⁻⁶⁸ The pathophysiological role of these immune cells is briefly set out below.

1.4.1 Innate Immune Cells

1.4.1.1 Microglia

Microglia are brain-resident immune cells of myeloid lineage. They are unique among cells in the CNS in that they do not develop from the neural ectoderm. Rather, they are mesenchymal in origin. They develop in the yolk sac in early embryogenesis and migrate to the brain rudiment prior to any differentiation among neural tissue.⁶⁹ There, they adopt the role of tissue-resident macrophages and deploy highly complex ramifications to survey the local environment. They are capable of diversified cytokine and chemokine production, phagocytosis and antigen presentation.⁷⁰ In addition to these obvious immune activities, they are also increasingly implicated in an important range of neurobiological functions, including early CNS development, myelin turnover and tissue repair.⁷¹

Microglia appear to play dichotomous roles in MS lesions, dependent on the context of their activation and immune polarisation state. For example, depletion of microglia⁷² or the inhibition of their activation⁷³ can improve outcomes of EAE. Furthermore, given their myeloid lineage, microglia are capable of efficient antigen presentation and activated MHC class II (MHC-II) positive microglia have been found in abundant numbers

in active MS lesions.^{36, 74} Conversely, microglia can also assume an anti-inflammatory role, akin to M2 macrophage polarisation. In this context, microglia can secrete factors such as brain-derived neurotrophic factor⁷⁵ and leukaemia inhibitory factor⁷⁶ that promote oligodendrocyte survival and remyelination.^{77, 78} As mentioned previously, microglia also appear to play a pivotal role in the clearance of myelin debris, a potential source of CNS auto-antigen. Finally, the most recently published genomic map for multiple sclerosis noted enrichment of MS genes within microglia but not in astrocytes or neurons, suggesting a key role for this cell type in MS susceptibility.²³

1.4.1.2 Macrophages

In the setting of autoimmune demyelination, degradation of the BBB allows peripheral monocytes to join microglia as the primary innate immune cell drivers of local inflammation. Their role in pathogenesis of central demyelination was demonstrated by the amelioration of the severity of EAE in a *CCR2* knock-out model which prevented the ingress of peripheral monocytes into the CNS.⁷⁹

Given their different lineage, it has been proposed that infiltrating macrophages and resident microglia may play very different roles in the process of demyelination. In a recent study that employed genetic labelling techniques, bone-marrow derived macrophages appeared to trigger demyelination and expressed a highly inflammatory transcriptome. In contradistinction, microglia-derived macrophages appeared to mediate debris removal and expressed a metabolically restricted gene profile.⁸⁰ Taken together, these data suggest that infiltrating macrophages may perpetuate maladaptive responses to central demyelination while resident microglia may either mediate tissue injury or repair depending on their activation state. This complex relationship will require further investigation if therapeutics targeting innate immune responses in MS are to prove efficacious.

1.4.1.3 Dendritic Cells

Dendritic cells are specialised antigen presenting cells (APC) of the innate immune system. They are intimately involved in antigen presentation to immature T cells. In this role, they are an important link between the innate and adaptive immune systems. While inactivated, dendritic cells survey their local environment by examining phagocytosed elements for self-compatibility. In this state they express low levels of stimulatory elements, with MHC-II largely sequestered in endosomes. However, when foreign or immunogenic material is encountered, MHC-II is shuttled to the cell surface and dendritic cells assume their role as highly efficient APCs capable of T cell co-stimulation.⁸¹⁻⁸³

Activated dendritic cells are found in MS lesions. Of particular interest, these cells were found to contain phagocytosed myelin and expressed co-stimulatory molecules in concert with CD8⁺ T cells.⁸⁴ Furthermore, in EAE models a discrete population of meningeal dendritic cells have been identified that are responsible for antigen presentation to infiltrating T cells. Dendritic cells also facilitated ingress of T cells across the BBB. Exogenous stimulation of this dendritic cell population resulted in more severe clinical phenotypes.⁸⁵

In contrast to this pro-inflammatory role, subsequent research has demonstrated that a discrete population of CD11b⁺CD11c⁺ dendritic cells manifest an anti-inflammatory phenotype. Mice injected with MOG₃₅₋₅₅ peptide developed less severe EAE following prior immunization with that peptide and demonstrated upregulation of CD11b⁺CD11c⁺ dendritic cells in the CNS and spleen. Furthermore, when these cells were co-cultured with T cells or lipopolysaccharide, they demonstrated immunoregulatory characteristics, including increased production of interleukin (IL) 10 and TGF-beta and suppressed IL-12 and NO. In addition, these cells inhibited the proliferation of MOG-specific T cells. Clinically, these animals pre-immunised with MOG₃₅₋₅₅ prior to EAE induction also demonstrated a markedly improved disease phenotype.⁸⁶

As with microglia, it appears that differing subpopulations and/or polarisation states of dendritic cells can yield either pro- or anti-inflammatory phenotypes. This distinction will be important in the development of therapeutics that aim to target this aspect of immune activation.

1.4.2 Adaptive Immune Cells

1.4.2.1 T Cells

The discovery that EAE could be induced in naïve animals by the transfer of activated anti-MOG CD4⁺ T cells led to the theory that MS was also a CD4⁺ T cell mediated disease.^{8, 37} Indeed, CD4⁺ T cells are found in active MS lesions. As with other immune cells, their role in autoimmune demyelination appears to be heavily influenced by polarisation state. Pro-inflammatory Th1 states are induced by IL-12 exposure and characterised by the production of interferon gamma (IFN γ), tissue necrosis factors alpha (TNF α) and beta (TNF β) and IL-2. Anti-inflammatory, Th2 states are induced by IL-4 exposure and characterised by the production of IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and transforming growth factor beta (TGF β). High levels of circulating IFN γ in MS patients appeared to corroborate experimental findings with EAE.⁸⁷ An important role of CD4⁺ T cells was also enhanced by genetic studies identifying HLA-DRB allele status as the strongest genetic risk factor for the development of MS.²³

However, the population of CD4⁺ T cells found in MS lesions is relatively small. Indeed, these cells are greatly outnumbered by MHC-I restricted CD8⁺ T cells.⁸⁸ Furthermore, clonal expansion of T cells in MS occurs predominantly in CD8⁺ lineages.⁵⁷ Importantly, neural structures targeted in MS, including neurons and glia, express MHC-I but not MHC-II.⁵⁸ Recent developments have also identified that Th17 pro-inflammatory T cells are abundant in MS patients and their presence appears positively correlated with relapse activity. This raises the possibility of targeting a novel and discrete T-cell population for therapeutic intervention.⁸⁹

Regulatory T cells (T_{reg}) are also important modulators of inflammatory demyelination. In a spontaneous model of EAE (sEAE) inactivation of regulatory populations of T cells resulted in a more severe disease phenotype. Conversely, the transfer of tolerogenic splenocytes into sEAE-susceptible mice resulted in the expansion of the T_{reg} population.⁹⁰ There are also apparent deficiencies in T_{reg} signalling in patients with MS compared to healthy controls, leading to impaired downregulation of myelin-specific autoreactive effector cells.⁹¹

1.4.2.2 B Cells

Although T cells have been the focus of most research efforts in MS, multiple lines of evidence support a role for B cells (and their immunoglobulins) in the pathogenesis of the disease. Indeed, the identification of clonally restricted antibodies in cerebrospinal fluid (CSF) remains a commonly used clinical test to support a diagnosis of MS.⁹²

B cells are capable of modulating immune responses in autoimmune demyelination by a number of mechanisms. By differentiating into plasma cells, B cells can secrete autoantibodies that opsonise myelin products and process these antigens for T cell activation and macrophage phagocytosis. Similarly, B cells are able to assume an antigen presentation role for autoreactive T cells. Finally, they are capable of secreting both pro- (IL-6, IL-12, $TNF\alpha$) and anti-inflammatory cytokines (IL-10) to either enhance or suppress immune responses.⁶

The involvement of antibody-mediated mechanism in autoimmune demyelination is supported the presence of IgG on the surface of microglia and macrophages engaged in myelin breakdown⁹³ and by co-deposition of IgG and activated complement fragments and complexes at the border of active MS lesions.⁹⁴ Perhaps the most compelling evidence regarding the role of B cells in the pathophysiology of MS comes from experience in the use of anti-B cell therapies to improve clinical outcomes. Rituximab and ocrelizumab are monoclonal antibodies that target the CD20 receptor on B cells. Both have demonstrated efficacy in reducing clinical relapses and lesion

accumulation.^{95, 96} Indeed, ocrelizumab was recently identified as the first therapeutic agent to potentially offer modest improvement in disability progression in patients with PPMS.⁹⁷

1.5 The Role of TAM Signalling in Central Demyelination

The TAMs are a family of receptor tyrosine kinases expressed by discrete cell types, including macrophages, microglia and dendritic cells. They are involved the homeostatic regulation of adult, fully differentiated tissues that are subject to constant intrinsic and environmental challenge.⁹⁸ The MERTK and AXL proteins are further involved in the regulation of innate immune responses and their dysfunction or deletion leads to various immune-related disorders.^{99, 100} A substantial body of evidence now indicates that the TAMs are critical determinants of the outcome of central demyelination. Large, genome-wide association studies have identified polymorphisms in the MERTK gene as risk factors for the development of MS.^{101, 102} Administration of exogenous GAS6 – a ligand which can activate all three TAM receptors – increases neuronal repair and improves outcomes in cuprizone-induced demyelination and EAE.^{103, 104} Conversely, loss of GAS6 leads to increased severity of cuprizone-induced central demyelination and delays disease recovery.^{105, 106} This highlights the potential for the development of TAM agonists in the treatment of MS.

1.5.1 The TAM Receptor

The TAM receptors appeared relatively late in evolution - they are not found in *Drosophila* or *C. elegans* species.¹⁰⁷ Early homologues were first identified in tunicated *Ciona* during an evolutionary period when type I and II cytokines first emerged, suggesting a role in the development of immune regulation.⁹⁸

The TAM receptors have a common extracellular structure that involves two Ig-like domains at the N terminus followed by two fibronectin type III (FNIII) like domains joined by a single-pass transmembrane domain to cytoplasmic tyrosine kinase (TK) domain.¹⁰⁸ Like all receptor tyrosine kinases (RTK), ligand binding causes dimerization with

subsequent downstream signalling and activation.¹⁰⁹ In addition to membrane-bound forms, all TAM receptors can also be subject to proteolytic cleavage, resulting in soluble receptors that can act as ligand decoys.^{110, 111}

The efficiency of TAM receptor signalling is greatly enhanced by the simultaneous binding of the TAM receptor ligands, GAS6 and Protein S (ProS), to phosphatidylserine. For example, GAS6 that is lacking the domain required for phosphatidylserine binding can bind to but not activate the AXL receptor and its activity on TYRO3 and MERTK receptors is greatly reduced.¹¹² This suggests that TAM receptor activation is largely dependent on the presence of phosphatidylserine, a ubiquitous component of cell membranes and a powerful phagocytosis signal expressed by apoptotic cells.

1.5.2 TAM Receptor Ligands

Two TAM receptor ligands have been identified – GAS6 and ProS. They enjoy approximately 45% sequence homology¹¹³ and possess similar domain structures, with an N-terminal Gla domain followed by four epidermal growth factor (EGF) like domains. The C-terminal ends contain two laminin G (LG) domains. Binding of both ligands to the TAM receptors is mediated by the LG1 domain which can contact both Ig-like domains on the receptor. The Gla domains of both ligands are post-translationally carboxylated in a vitamin-K dependent manner.¹¹⁴ This step is essential as carboxylated glutamic acid in the Gla domain is required for Ca²⁺-dependent binding to phosphatidylserine.¹¹⁵

ProS is well characterised for its non-TAM dependent role as a regulator of blood coagulation. It is a co-factor for activated Protein C, where it enhances the proteolytic inactivation of Factors Va and VIIIa by Protein C, leading to downregulation of the pro-coagulant effect of these factors.¹¹⁶ Not surprisingly, given its role in modulating procoagulant effects, ProS null mice are embryonic lethal due to coagulopathy and haemorrhage. These animals also display severe vascular dysgenesis, which may reflect the loss of ProS as a TAM receptor ligand.¹¹⁷ The *GAS6* gene was initially identified as one of several upregulated in the context of serum-starvation induced growth arrest of

NIH3T3 fibroblasts, hence its name Growth Arrest-Specific 6.¹¹³ It has since been shown to play a role in the proliferation and survival of multiple cell types, including oligodendroglia.^{105, 118} Despite its homology with ProS, GAS6 does not play a role in the coagulation cascade due to the lack of thrombin-sensitive cleavage sites.¹¹⁹ GAS6 null mice demonstrate impaired platelet aggregation with a tendency to decreased thromboses but are otherwise phenotypically normal under physiological conditions.¹²⁰ While GAS6 is capable of binding to all three TAM receptors, ProS does not bind to AXL.¹²¹

1.5.3 Downstream Functions of TAM Activation

TAM receptor signalling has been implicated a wide range of cellular functions, including cell survival and proliferation, immune regulation and phagocytosis. TAM signalling promotes the survival of vascular smooth muscle cells,¹²² fibroblasts,¹²³ testicular cells¹²⁴ and macrophages.¹²⁵ Within the nervous system, TAM receptors appear to exert survival effects on hippocampal neurons¹²⁶ and Schwann cells,¹²⁷ in addition to cultured oligodendrocytes.¹¹⁸ Laboratory colleagues have demonstrated that the TAM receptor TYRO3 regulates developmental myelination and myelin thickness, independent of any influence on oligodendrocyte precursor maturation or oligodendrocyte or microglial function.¹²⁸⁻¹³⁰

In addition to survival signals, TAM receptors also promote proliferation of distinct cell types and the dysregulation of this function is implicated in the development of a broad range of human malignancies.¹³¹ In addition, the TAM receptor MERTK is expressed by innate immune cells, including macrophages, microglia and dendritic cells. In these cells, MERTK is essential for the homeostatic regulation of adult, fully differentiated tissues that are subject to constant challenge and regular renewal.⁹⁸

The advent of TAM receptor knock-out animals allowed for the elucidation of their role in immune regulation. TAM receptors are found in cells of both the innate¹⁰⁰ and adaptive immune systems.¹³² Silencing their activity results in splenomegaly and

constitutive activation of B and T cells with widespread autoimmune phenotypes involving a diversity of tissue. In particular, antibodies against auto-antigens are promoted, including double-stranded DNA, collagens and phospholipids.¹⁰⁰

In addition to an autoimmune phenotype, TAM triple knock-out (TKO) mutant mice also develop, *inter alia*, male infertility and blindness. These conditions reflect the essential role TAMs play in modulating the phagocytosis of apoptotic cells. Testicular degeneration occurs in adult male TAM TKO mice approximately one week after spermatogenesis. Although the testicles develop normally in these animals, they are not able to phagocytose apoptotic germ cells in the seminiferous tubules. In wild type mice, Sertoli cells phagocytose these cells in a TAM-receptor dependent manner which allows for clearance of cellular debris and efficient sperm production. In the absence of TAM receptors, apoptotic germ cells are not cleared which leads to local congestion and subsequent death of all germ cells.¹³³ Similarly, under physiological conditions cells of the retinal pigment epithelia (RPE) perform homeostatic phagocytosis of the distal ends of photoreceptors, which are subject to oxidative stress and require daily pruning and regeneration. This function is mediated by MERTK signalling and TKO mice are not able to maintain the length of photoreceptor outer segments. Due to retinal crowding, photoreceptors undergo apoptosis and these mice experience blindness after approximately one week.¹²¹ There is a direct human corollary with this phenomenon. A rare form of inherited retinitis pigmentosa has been identified due to mutations in the human gene encoding MERTK.^{134, 135}

1.5.4 TAM Signalling in Autoimmunity

The TAM receptors influence immune function in several important ways. Firstly, in dendritic cells, they influence antigen presentation and thereby modulate the adaptive immune response. The TAMs also induce phagocytosis of apoptotic cells by macrophages and microglia^{136, 137} an activity that is dependent upon ligand bridging between the receptors and phosphatidylserine on the surface of apoptotic cells. This phagocytic activity has been shown to be important for limiting the epitope spreading

of autoantigens in animal models of SLE.¹³⁸ The TAMs can also transform the activity of mononuclear cells from a pro-inflammatory (M1) towards an anti-inflammatory (M2) oriented phenotype, in particular by modifying the signalling response to type 1 interferons.¹³⁹

My parent laboratory was the first group to link TAMs to MS and demonstrated that TAM signalling influences outcomes in experimental models of demyelination and remyelination in the CNS. Mice deficient in GAS6 exhibit compromised oligodendrocyte survival, worse demyelination, fewer myelinated axons and potentiated microglial activation when subjected to demyelination induced by cuprizone.^{105, 106} In addition, in the context of human disease, this group was the first to show that MERTK is a susceptibility gene for MS.¹⁰¹ Upregulated expression of MERTK has been found within MS plaques, where it is suggested that inhibition of protective GAS6 receptor signalling is caused by over-expression of soluble MERTK and AXL. They may both act as non-signalling decoy receptors, thereby prolonging lesion activity.¹⁴⁰

1.6 The Scope of the Present Study

The innate immune system plays an integral role in the recruitment of immune cells to sites of infection and inflammation through the production of chemokines. It is also implicated in the modulation of cellular function at the site of pathology via cytokine release. Unlike the adaptive immune system, it does not confer long-lasting or protective immunity, although some innate immune cells, in particular dendritic cells, interact directly with the adaptive immune system and mediate antigen presentation. Other innate immune cells, including central microglia and peripheral macrophages, play important roles in resolving inflammation and facilitating tissue repair. They also maintain tissue homeostasis through the phagocytosis of debris, pathogens and dead cells.^{105, 106, 141} The innate immune system can become dysregulated and contribute to the development of human disease, including autoimmune conditions such as Systemic Lupus Erythematosus (SLE).¹⁴²

Two key cells of the innate immune system, macrophages and microglia, play important roles in disease pathogenesis and tissue repair. Intimate anatomical associations between microglia and other neural cells have been identified, suggesting that microglia could be involved in both the disruption of axons and myelin.¹⁴³ In rodent models of MS, there is evidence of the disease-promoting effects of monocytes/macrophages^{8, 144} with these cells exerting a critical role in the effector phases of disease. Human MS and rodent EAE lesions also contain phagocytically active microglia/macrophages that contain myelin degradation products. These observations suggest either direct oligodendrocyte attack, or conversely, a role for the innate immune system in neuronal repair. Current evidence strongly implicates innate immune activation and the phagocytosis of myelin debris as a necessary prerequisite for remyelination.^{8, 145} Phagocytic (M2) macrophages also ameliorate inflammatory demyelination in animal models.¹⁴⁶

The extent to which MERTK signalling in CNS demyelination is mediated by central innate immune cells (microglia), as opposed to peripheral innate immune cells (macrophages) remains uncertain and has implications for the development and delivery of therapeutics targeting the TAMs. The influence of MERTK signalling on systemic innate immune cell-mediated antigen presentation (by dendritic cells) and the consequences for the adaptive immune response also requires investigation, particularly in the context of inflammatory demyelinating disease. The EAE disease model is particularly well suited to these investigations as it involves activation of adaptive and innate immune cells. Only by characterising the immune interactions driven by MERTK expression can steps be taken to develop therapeutics that manipulate its activity. Accordingly, my research is based on the following hypotheses and experimental aims:

- Hypothesis One: MERTK expressed by dendritic cells modulates inflammatory responses in experimental autoimmune encephalomyelitis (EAE).

- Aim One: To investigate the effect of murine MERTK deletion in dendritic cells on the outcomes of inflammatory central demyelinating disease after induction of EAE and to investigate the effect of murine MERTK deletion in microglia on the outcomes of central demyelinating disease after cuprizone intoxication.

- Hypothesis Two: Human monocyte and/or dendritic cell expression of MERTK correlates with MS disease activity.

- Aim Two: To compare levels of MERTK expression in MS patients during periods of disease relapse with disease quiescence, using healthy subjects as controls and to correlate with an analysis of the *MERTK* single nucleotide polymorphism rs7422195 status.

The outcome of these investigations will contribute to the growing body of evidence that implicates the TAM receptor family in the development of autoimmune diseases in general and MS in particular. As TAM receptors are essential for a wide range of physiological processes, accurate characterisation of their role in inflammatory demyelinating conditions will be essential if their expression is to be safely enhanced or abrogated in pursuit of therapeutic benefits for patients.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Macquarie Park, Australia) unless specified otherwise. All primers were prepared by IDT GeneWorks (Thebarton, Australia).

2.2 Solutions

Tail lysis buffer

100mM Tris-HCl, 5mM EDTA, 200mM NaCl, 0.2% SDS (w/v) in MilliQ H₂O.

Phosphate Buffered Solution (PBS)

10mM Na₂HPO₄, 1.8mM KH₂PO₄, 137mM NaCl, 2.7mM KCl in MilliQ H₂O.

Mouse Tonicity Phosphate Buffered Solution (MT-PBS)

16.3mM Na₂HPO₄, 62.7mM NaH₂PO₄, 148mM NaCl in MilliQ H₂O.

4% paraformaldehyde (PFA)

4% PFA (w/v) (Merck KGaA, Darmstadt, Germany) in MT-PBS.

TAE buffer

40mM Tris-acetate, 1mM EDTA in MilliQ H₂O. 41

FACS buffer

2mM EDTA, 2% Foetal Calf Serum (ThermoFisher Scientific, Waltham, MA) in DPBS (ThermoFisher Scientific, Waltham, MA).

Red blood cell lysis buffer

154.4mM NH₄Cl, 10mM KHCO₃, 1mM EDTA in MilliQ H₂O.

2.3 Animal Resources

C57BL/6 wild-type mice were obtained from Animal Resources Centre (Canning Vale, Australia). The *Gas6*^{-/-} mouse line was previously established at the Florey Institute for Neuroscience and Mental Health and was originally a kind gift from Prof. Peter Carmeliet of the Vesalius Research Center, Leuven, Belgium. Initial generation of the *Gas6*^{-/-} mouse line is detailed by Angelillo-Scherrer et al.¹²⁰

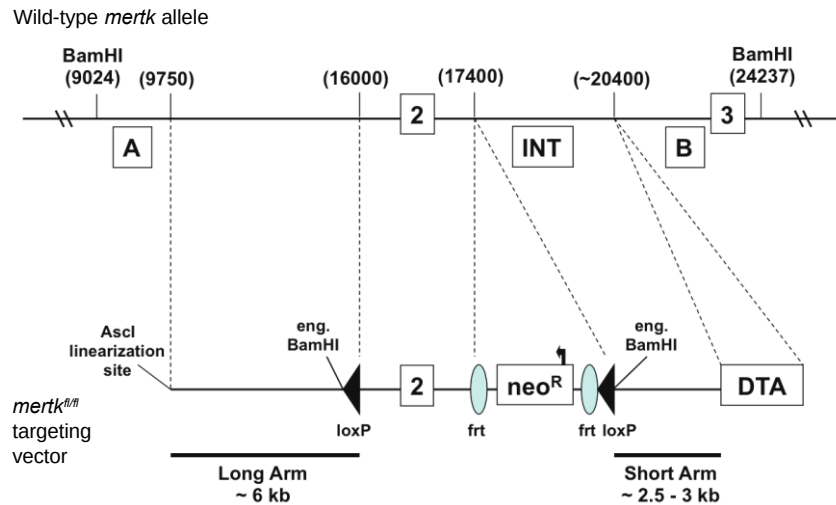
The CD11c-Cre mouse line was a kind gift of Dr. Marc Pellegrini of the Walter and Eliza Hall Institute of Medical Research, Australia. Initial generation of the CD11c-Cre mouse line is detailed in Caton et al.¹⁴⁷ To generate the appropriate CD11c-Cre/*Mertk* genotypes for experimentation, the breeding strategy outlined in Figure 2.1 was employed.

2.3.1 Construction of the *Mertk*^{fl/fl} Mouse

The *Mertk*^{fl/fl} mouse was created by Dr. Renate Lewis at the Transgenic Vectors Core of the Hope Center for Neurological Disorders, Department of Neurology, Washington University School of Medicine, St. Louis, MO. Mice from a 129/SvEv background were used for embryonic stem cell manipulation. Exon 2 of the *Mertk* gene was conditionally deleted by the introduction of early stop codons, thereby excluding the chance of dominant negative effects as the result of a kinase dead MERTK receptor. Five prime to exon 2, a loxP site was introduced by homologous recombination into a non-conserved region with a mouse 129/SvEv bacterial artificial chromosome (BAC) containing the *Mertk* genomic locus. A *frt*-flanked pGK-neomycin expression cassette was also introduced 3' of exon 2 into a non-conserved region in order to facilitate positive selection in embryonic stem (ES) cells. A second, flanking loxP site was also introduced. Subsequently, an approximately 10.6 kb fragment, including the floxed region and the *frt* flanked pGK-neomycin cassette, was generated from the modified BAC via gap repair in a vector containing a diphtheria toxin cassette. The diphtheria toxin cassette provided negative selection in ES cells. *Ascl* was used in order to linearise the targeting vector for ES cell electroporation (Fig. 2.1a). The Mouse Genetics Core, Washington University

School of Medicine, St. Louis, MO completed the electroporation, selection of ES cells, microinjection and subsequent generation of *Mertk^{fl/fl}* chimeric mice. *Mertk^{fl/fl}*, *Mertk^{fl/wt}* and *Mertk^{wt/wt}* mice were born at expected Mendelian frequencies. No phenotypic or behavioural differences were observed in these animals compared to wild-type 129/SvEv mice.

a.



b.

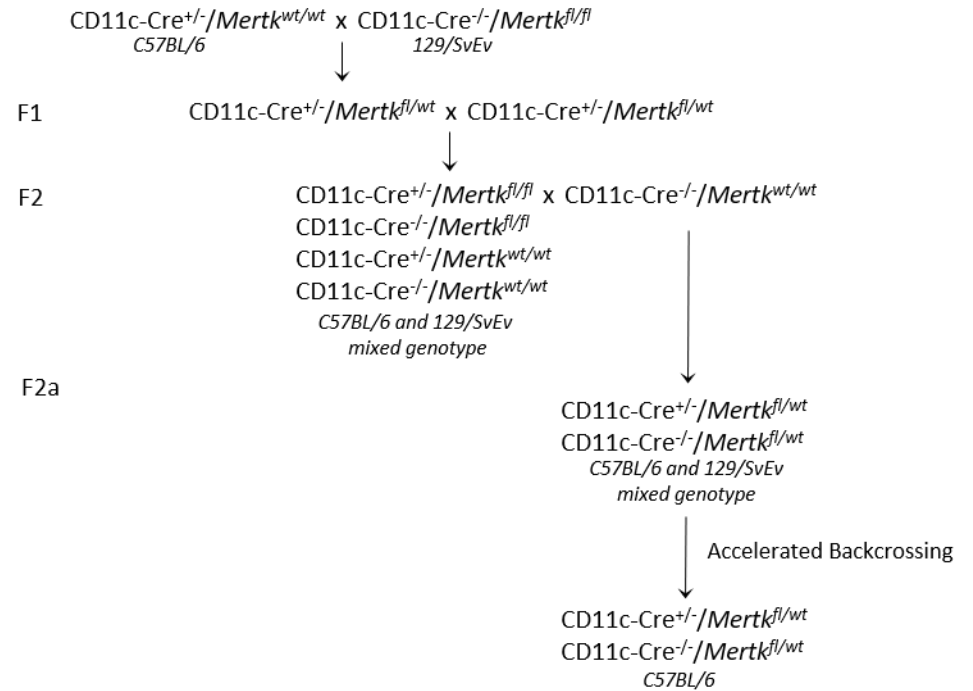


Figure 2.1. Construction of the *Mertk* conditional knock out mouse **a.** Exon 2 of the *Mertk* gene was targeted for conditional deletion by the use of early stop codons. Exon 2 was flanked by loxP sites and pGK-neomycin (neo^R) and diphtheria toxin (DTA) cassettes were introduced for positive and negative selection of ES cells, respectively; **b.** CD11c-Cre/*Mertk* breeding strategy. CD11c-Cre^{+/-} mice on a C57BL/6 background were crossed with *Mertk*^{fl/fl} mice on a 129/SvEv background to produce the F1 generation. However, as outlined, CD11c-Cre^{+/-}/*Mertk*^{fl/fl} did not breed at Mendelian frequencies. Cre heterozygous mice from the F1 generation were then inbred to produce the F2 generation for experimentation. CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice were to be crossed with CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice to produce the F2a generation for heterozygous deletion experiments. CD11c-Cre^{+/-}/*Mertk*^{fl/wt} were then bred with marker-assisted backcrossing to yield animals on a uniform C57BL/6 background.

2.3.2 Generation of CD11c-Cre^{+/-}/Mertk^{fl/fl} and CD11c-Cre^{+/-}/Mertk^{fl/wt} Mice

Mertk was conditionally deleted from CD11c⁺ cells by crossing *Mertk*^{fl/fl} mice with mice expressing Cre under regulatory control of the CD11c promoter. CD11c-Cre has been reported to drive deletion from over 95% of dendritic cells with minimal deletion from granulocytes and adaptive immune cells.¹⁴⁷ As the CD11c-Cre line was on a C57BL/6 background and the *Mertk*^{fl/fl} line on a 129/SvEv background, double transgenic mice were initially bred on a C57BL/6 and 129/SvEv background. Subsequently, marker-assisted accelerated backcrossing was pursued to yield animals on a predominantly C57BL/6 background (Fig. 2.1b). In particular, mixed background animals were used to examine TAM receptor and ligand expression under physiological conditions and during experimental autoimmune encephalomyelitis (EAE). Early EAE cohorts performed by Dr. Gerry Ma were also undertaken in mixed background animals. Later cohorts of heterozygous (CD11c-Cre^{+/-}/Mertk^{fl/wt}) deleted animals and all cohorts of homozygous (CD11c-Cre^{+/-}/Mertk^{fl/fl}) deleted animals were performed in fully backcrossed animals.

The resulting progeny of CD11c-Cre^{+/-}/Mertk^{fl/fl}; CD11c-Cre^{-/-}/Mertk^{fl/fl}; CD11c-Cre^{+/-}/Mertk^{fl/wt}; CD11c-Cre^{+/-}/Mertk^{wt/wt}; and CD11c-Cre^{-/-}/Mertk^{wt/wt} demonstrated no abnormal phenotype and were behaviourally indistinguishable from wild-type mice. CD11c-Cre^{-/-}/Mertk^{wt/wt} and CD11c-Cre^{+/-}/Mertk^{fl/fl} mice were not bred at expected Mendelian frequencies. Given the normal phenotype observed in those progenies that were obtained, I hypothesise that the CD11c-Cre transgene is likely incorporated on the same chromosome as *Mertk* (chromosome 2) with only occasional CD11c-Cre^{+/-}/Mertk^{fl/fl} and CD11c-Cre^{-/-}/Mertk^{wt/wt} animals produced through random recombination. Accordingly, a single female CD11c-Cre^{+/-}/Mertk^{fl/fl} was crossed with a male CD11c-Cre^{-/-}/Mertk^{fl/fl} animal for all experiments that examined EAE in homozygous deleted animals.

In order to recapitulate EAE experiments in animals with a mixed C57BL/6 and 129/SvEv background, male and female CD11c-Cre^{+/-}/Mertk^{fl/fl} animals on a C57BL/6 background were crossed with WT 129/SvEv animals to yield CD11c-Cre^{+/-}/Mertk^{fl/wt} and CD11c-Cre⁻

$l^{-}/Mertk^{fl/wt}$ progeny. These first generation animals were used for all EAE experiments involving mixed C57BL/6 and 129/SvEv backgrounds.

2.4 Experimental Autoimmune Encephalomyelitis

2.4.1 Induction of Experimental Autoimmune Encephalomyelitis (EAE)

EAE was induced as previously described.⁷⁷ Briefly, eight- to twelve-week-old mice were immunised subcutaneously with a myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅) (MEVGWYRSPFSRVVHLYRNGK)(Mimotopes, Clayton, Australia) diluted to 1mg/ml in MT-PBS and emulsified in 15ml Complete Freund's Adjuvant containing 100mg of desiccated *M. tuberculosis* (both from Becton Dickinson, Franklin Lakes, NJ). In order to form an emulsion for antigen delivery, the solution was mixed at 10,000RPM for 100 seconds on a Mini-Beadbeater (Biospec Products, Bartlesville, OK). Subsequently, 250μL of MOG/CFA/TB emulsion was delivered to each animal by subcutaneous injection of 100μL to both flanks and 50μL to the base of tail. In addition, mice received intra-peritoneal injections of 400ng pertussis toxin (Sapphire Bioscience, Redfern, Australia) on days 0 and 3 post-EAE induction.

2.4.2 EAE Clinical Severity Index

An EAE clinical severity index was recorded daily using a standard nine-point paraplegia scale with 0.5-point increments, as previously described.¹⁴⁸ Mice that reached grade 3.5 were killed in accordance with ethical requirements. The last documented score of euthanized animals was carried forward for statistical analysis. EAE experiments were terminated at varying time points to perform analyses during the effector phase of immune-mediated demyelination (days 8-12 post-induction), at disease peak (days 15-21 post-induction) or during recovery (day 30 post-induction). Most EAE experiments were terminated at day 18. This time point typically reflects disease peak and was chosen so that PCR, flow cytometric and immunohistochemical analyses would capture physiological conditions at the peak of immune activation. The typical progression of MOG₃₅₋₅₅ EAE is set out in Figure 2.2. Mice were cohorted in mixed genotypes and scoring was performed by researchers blinded to genotype.

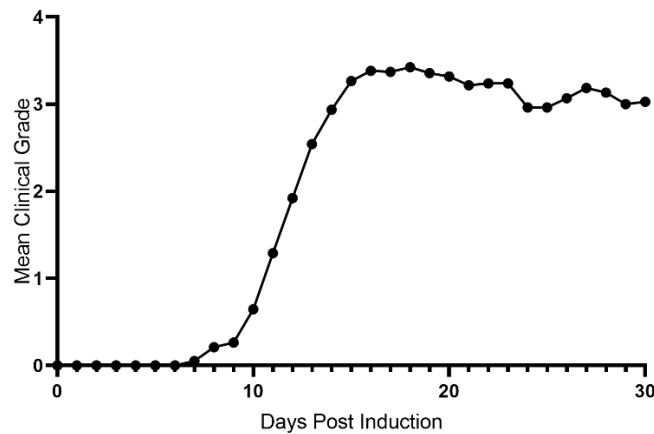


Figure 2.2. Typical progression of MOG₃₅₋₅₅ EAE. Clinical severity was assessed on an ordinal paraplegia scale as follows: 0: healthy; 1.0: tail weakness; 1.5: tail weakness and hindlimb weakness; 2.0: complete tail paralysis; 2.25: complete tail paralysis and some hindlimb weakness; 2.5: complete tail paralysis and severe hindlimb weakness; 2.75: complete tail paralysis and paralysis of one hindlimb; 3.0: complete tail and hindlimb paralysis; 3.5: complete tail and hindlimb paralysis with forelimb weakness and loss of righting reflex or signs of urinary or faecal incontinence; 4.0: death. Animals that were graded at 3.0 for more than three days or 3.5 for one day were euthanised, in keeping with Ethics Committee requirements. Samples were collected at time points corresponding to the pre-symptomatic (effector) phase (day 8-12); established disease (day 15); disease peak (day 18-21); and disease recovery (day 30).

2.5 Cuprizone Induced Central Demyelination

Cuprizone mediated demyelination was induced by feeding 8–10-week-old CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice pelleted feed (Barastoc, Pakenham, Australia) containing 0.2% cuprizone (w/w: bis-cyclohexanone-oxaldihydrazone) for three weeks. During this period, feed was refreshed on alternate days. Animals were examined for clinical distress and were weighed on alternate days. CD11c-Cre^{-/-}/*Mertk*^{fl/fl} animals were used as controls. For each cohort, equal numbers of male and female mice were subjected to cuprizone challenge.

2.6 Histology and Immunohistochemistry

2.6.1 Tissue Collection

Mice were anaesthetized by intra-peritoneal injection of sodium pentobarbitone (100mg/kg, Virbac, Wetherill Park, Australia) in MT-PBS and then perfused intracardially with 20ml MT-PBS at 37°C followed by 20ml of ice cold 4% (w/v) paraformaldehyde (Merck KGaA, Darmstadt, Germany). For animals induced with EAE, spinal cords were dissected from the vertebral column and the lumbar expansion was excised. For animals

exposed to cuprizone intoxication, the head was removed and the brain dissected *en bloc*. Harvested tissue was post-fixed for 30 minutes in ice cold 4% PFA and then stored overnight at 4°C in 20% sucrose (Amresco, Solon, OH) in MT-PBS for cryoprotection. Tissue was then embedded in Tissue-Tek optimum cutting temperature compound (Sakura Fintech, Tokyo, Japan) and frozen on dry ice. For lumbar expansions, axial sections were cut using a cryostat at 10µm intervals and collected onto Superfrost++ slides (Menzel Glass, Braunschweig, Germany). Whole brains were sectioned in coronal orientation and the rostral corpus callosum was identified as close to Bregma 0.38 as possible.

2.6.2 Immunohistochemistry

Frozen sections were rehydrated in MT-PBS and blocked for one hour in 10% normal goat serum (Millipore, Burlington, MA) and 0.3% Triton X-100 (v/v) in MT-PBS (blocking solution). Tissue was stained with primary antibodies diluted in blocking solution overnight at room temperature. MAC3 visualisation required antigen retrieval, performed by immersing samples in methanol for five minutes at room temperature prior to incubation with primary antibodies. Samples were then washed and incubated with fluorophore-conjugated secondary antibodies diluted in blocking solution for one hour. Sections were mounted using Dako Fluorescence Mounting Media (Agilent Technologies, Santa Clara, CA).

2.6.3 Quantification of Immunohistochemical Findings

Following immunohistochemical staining, sections were imaged using an Axioplan microscope (Carl Zeiss, Oberkochen, Germany). Images were then opened in Adobe Photoshop CC 2018 (Adobe Systems, San Jose, CA) and for EAE specimens the dorsal column of the lumbar spinal cord was identified and outlined. For cuprizone specimens the rostral corpus callosum was identified and outlined. All cells of interest in outlined areas were counted. The area of the dorsal column or rostral corpus callosum was calculated using ImageJ version 1.52a (National Institutes of Health, Bethesda, MD) and

all results expressed as cells/mm². All counts were performed by a researcher blind to animal sex and genotype.

Table 2.1: Primary Antibodies for Immunohistochemistry

Target Antigen	Antibody Abbreviation	Host Subtype	Dilution	Source
CD3	CD3	Rat	1:200	eBioscience, San Diego, CA, Clone 17a2
CD11c	CD11c	Armenian Hamster	1:50	Novus Biologicals, Centennial, CO, clone AP-MAB0814
Ionised calcium-binding adapter molecule 1	IBA1	Polyclonal Rabbit Ig	1:1000	FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, clone NCNP24
Surface antigen MAC3	MAC3	Rabbit	1:100	Biolegend, San Diego, CA, clone M3/84
Olig2	Olig2	Rabbit IgG	1:100	IBL International, Cat# JP18953
α -Type platelet-derived growth factor receptor	PDGFR α	Polyclonal Goat IgG	1:150	R&D Systems, Cat# AF1062
2',3'-cyclic nucleotide 3'-phosphodiesterase	RIP	Monoclonal Mouse IgG	1:1000	Millipore Cat# MAB1580
Cell nuclei	DAPI		1:1000	Hoechst 33342, Thermo Fisher Scientific, Waltham, MA; 1:10,000

Table 2.2: Secondary Antibodies for Immunohistochemistry

Antibody	Conjugate	Dilution	Source
Polyclonal goat anti-rabbit	FITC	1:200	Jackson ImmunoResearch, West Grove, PA, CAT# 111-095-003
Polyclonal goat anti-rabbit	Alexa 594	1:200	Jackson ImmunoResearch, West Grove, PA, CAT# 111-585-003
Polyclonal goat anti-rabbit	TRITC	1:200	Jackson ImmunoResearch, West Grove, PA, CAT# 111-025-144
Polyclonal goat anti-rat	Alexa 488	1:200	Biolegend, San Diego, CA, Cat# 405418
Polyclonal goat anti-Armenian hamster	Alexa-594	1:50	Biolegend, San Diego, CA, Cat# 405512
Polyclonal goat anti-mouse	FITC	1:200	Jackson ImmunoResearch, West Grove, PA, CAT# 111-095-003
Polyclonal donkey anti-goat	TRITC	1:200	Jackson ImmunoResearch, West Grove, PA, CAT# 702-025-003

2.7 Flow cytometric analysis and fluorescent-activated cell sorting

2.7.1 Tissue Collection and Preparation

Mice were killed by injection of sodium pentobarbitone in MT-PBS. The spleen, thymus and/or inguinal lymph nodes were removed by dissection while bone marrow was flushed from both femurs with 5 mL of fluorescent-activated cell sorting (FACS) buffer from a syringe tipped with a 24-gauge needle. In order to obtain single cell suspensions from spleen, thymus and lymph nodes, tissue was gently ground using the plunger of a 3ml syringe through a 40µm filter, using FACS buffer (D-PBS with 2 mmol EDTA and 2% foetal calf serum) to wash the cells through the filter. A single suspension of bone marrow cells was made by repeated pipetting and passage through a 40µm filter.

After obtaining single cell suspensions, cells were pelleted at 300g for 10 minutes. Where necessary to lyse red blood cells, cell suspensions were resuspended in 5ml of red blood cell lysis buffer (Miltenyi Biotech, Bergisch Gladbach, Germany) and incubated for 7 minutes at room temperature. To stop lysis, 5ml of PBS was added to the suspension and cells were pelleted again by centrifugation at 300g for 10 minutes and subsequently resuspended in FACS buffer. After counting, cells were resuspended in 90µL FACS buffer with 10µL of mouse Fc receptor blocker (Miltenyi Biotech, Bergisch Gladbach, Germany) per 10^7 cells and incubated at 4°C for 10 minutes.

2.7.2 Flow Cytometry Analysis

Prior to flow cytometric analysis, cells were washed using FACS buffer, resuspended and incubated with fluorophore-conjugated antibodies for 30 minutes at 4°C. Following incubation, cells were pelleted and washed with FACS buffer and then resuspended in a volume suitable for flow cytometric analysis. Flow cytometry was performed on either a CyAn ADP or CytoFLEX S flow cytometer (both from Beckman Coulter, Pasadena, CA). Median fluorescence intensity and mean proportion $\pm$ SEM were performed using either FlowJo 9 (FlowJo, Ashland, OR) or FSC Express 6/7 Research Edition (De Novo Software, Glendale, CA).

Table 2.3: Conjugated Antibodies for Flow Cytometry

Antibody Target	Order Number (Miltenyi Biotec, Bergisch Gladbach, Germany)
Anti-CD4-APC	Order No. 130-109-415
Anti-CD4-FITC	Order No. 130-091-608
Anti-CD8-PE	Order No. 130-091-603
Anti-CD11b-PE	Order No. 130-091-240
Anti-CD11c-FITC	Order No. 130-102-466
Anti-Mertk-APC	Order No. 130-107-479
REA-APC	Order No. 130-104-630

2.7.3 Fluorescent-Activated Cell Sorting

For fluorescent-activated cell sorting, prepared splenocytes were incubated at 4°C for 15 minutes with a custom negative-selection, depletion solution based on reagents from Miltenyi Biotec (Order Nos. 130-091-262 and 130-091-169), consisting of magnetic MicroBeads specific for CD4 (T_H cells), CD19 (B cells), Ter119 (erythrocytes), CD45R (B cells) and CD49b (NK cells) at a concentration of 1:9 per MicroBead. Following washing, unwanted cells were depleted by passing the cell suspension through a magnetic LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) and collecting the flow-through. Next, the depleted flow-through splenocytes were labelled for positive selection with fluorophore-conjugated antibodies specific for mouse CD11b and CD11c (Miltenyi Biotec, Bergisch Gladbach, Germany) by resuspending splenocytes in FACS buffer containing CD11b-PE (1:200) and CD11c-FITC (1:100) and incubating at 4°C for 30 minutes. Splenocytes were then resuspended in FACS buffer and sorted at the Melbourne Brain Centre Flow Cytometry Facility on a FACS Aria III (BD Bioscience, San Jose, CA). For dendritic cell analysis, all CD11c⁺ and CD11c⁺CD11b⁺ cells were isolated.

2.8 RNA Analysis

2.8.1 RNA Isolation and RT-PCR

Mice were killed by intra-peritoneal injection of sodium pentobarbitone in MT-PBS and spleens and spinal cords were dissected. The lumbar expansion of the spinal cord was identified and excised. Spleens and lumbar expansions were manually disrupted in a

glass tissue homogenizer in the presence of QIAzol reagent (QIAGEN, Venlo, The Netherlands). Subsequently, RNA was isolated using the RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands) in accordance with the manufacturer's instructions. This process includes a step to remove genomic DNA using a proprietary eliminator. For cells sorted by FACS (CD11b^{+ve}, CD11c^{+ve}) on column DNase digestion was performed during the initial RNA extraction process using the RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. Concentration and purity of RNA was quantified using a Nanodrop spectrophotometer (Biolab Scientific, Mumbai, India).

To generate cDNA, 1µg of RNA was reverse transcribed using the TaqMan reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) with random hexamers. Quantitative PCR was performed using a SYBR Green master mix (Thermo Fisher Scientific, Waltham, MA) on a ViiA 7 Real Time PCR System (Applied Biosystems, Foster City, CA).

Table 2.4: Quantitative PCR Reactions

Machine	Reagents	Thermal Cycler Conditions
7500 Fast	1x SYBR Green Master Mix 0.8µm Forward Primer 0.8µm Reverse Primer cDNA: 5µl Total volume: 25µl	50°C - 2 min 95°C - 10 min 60°C - 1 min } x45
ViiA 7	1x SYBR Green Master Mix 0.8µm Forward Primer 0.8µm Reverse Primer cDNA: 5µl Total volume: 25µl	50°C - 2 min 95°C - 10 min 60°C - 1 min } x45

Table 2.5: Quantitative PCR Primers

Gene	Primers (5' – 3')
18s	Forward 5'CGGCTACCACATCCAAGGAA3' Reverse 5'GCTGGAATTACCGCGGCT3' or Forward 5'CGAACGGTCTGCCCTATCAACTT3' Reverse 5'ACCCGTGGTCACCATGGTA3'
Axl	Forward 5'TCTAATGTTGGCCTGATCTTCGT3' Reverse 5'GCCTCTGTGACTTAGATGCTGAG3'
GAS6	Forward 5'GCTGCAGCTTCGGTACAATG3' Reverse 5'ACATGCCGTGGTTGATGGTT3'
Mertk	Forward 5'CTGCCGAGAAGTGGGAAGAG3' Reverse 5'CCCTGGTAAAGGCCCTGAAA3' or Forward 5'GACGGTGTCCAAGGGGTGTACAT3; Reverse 5'CTGTCTGTTTGAGGATATGGACTTCAG3'
ΔMertk (Mertk ^{fl/fl})	Forward 5'GGTTCTGGCCCCACTGCTA3' Reverse 5'TCTTACAGAAGTACGACCCATTGTCT3'
ProS	Forward 5'TTTGATGATAGTGCATTGCAAGTG3' Reverse 5'CGTCTGATGCACCCCTGAA3'
Tyro3	Forward 5'TGGCTGAGCTGCTCCTACTTTA3' Reverse 5'TGGGCAGTGCTGAGTTCCA3'

2.9 Statistical Analysis – Mouse Experiments

All statistical tests were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). A *p*-value of <0.05 was considered statistically significant. Non-parametric statistical analyses were conducted to determine statistical significance in EAE

experiments. The Mann-Whitney U test was used to analyse experiments involving two groups, while the Kruskal-Wallis test was used to analyse experiments involving more than two groups. The Log-rank (Mantel- Cox) test was used to compare disease incidence. Results are expressed as mean clinical grade $\pm$ SEM. For flow cytometric and immunofluorescent analyses, the Student's T test was used to compare groups. Results are expressed as mean proportion $\pm$ SEM. For gene expression analysis, one-way ANOVA analysis with Tukey's multiple comparisons test was used to determine statistical significance. Results are expressed as mean relative expression $\pm$ SEM. Within-grade analysis via one-way ANOVA was used to analyse the relationship between immune cell infiltrates and EAE clinical severity index.

2.10 Animal Ethics

All animal experiments were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee and conducted according to National Health and Medical Research Council (Australia) guidelines, with all appropriate effort made to minimise animal suffering.

2.11 Human Peripheral Blood Monocyte Analysis

2.11.1 Isolation of Human Peripheral Blood Monocytes

Human blood samples were collected using 10ml Vacutainer plastic whole blood collection tubes with spray-coated K2EDTA (Cat. No. 368589, Becton Dickinson, Franklin Lakes, NJ). Blood samples were stored at room temperature and peripheral blood mononuclear cell (PBMC) isolation was performed as soon as possible following collection, but in all cases within 24 hours.

Approximately 100 mls of whole blood was collected from each study participant at each time point. Samples were diluted 1:1 with room temperature FACS buffer in 50 mls centrifugation tubes. Diluted samples were mixed by gentle pipetting prior to transfer to 50 mls SepMate PBMC Isolation Tubes (STEMCELL Technologies, Vancouver, BC), preloaded with 15 mls Histopaque-1077 sterile density gradient media (Sigma-Aldrich,

Macquarie Park, Australia) at room temperature. SepMate PBMC Isolation Tubes were then centrifuged at 1200G for ten minutes with the brake on. The supernatant (approximately 35 mls) was subsequently transferred to clean 50 mls centrifugation tubes and additional FACS buffer was added to bring the total volume to 50 mls. The samples were centrifuged again at 400G for ten minutes with the brake on. In order to maximise PBMC yields, the supernatant from this second centrifugation step was collected in separate centrifugation tubes which were again spun at 400G for ten minutes. During that step, the pelleted PBMCs were resuspended in 2 mls FACS buffer and maintained at room temperature. At the completion of the second PBMC centrifugation step, the supernatant was discarded and 1 ml FACS buffer was added to each tube. The pelleted PBMCs from this step were resuspended and combined with the PBMCs from the first collection step. All samples were then consolidated into a single centrifugation tube to which further FACS buffer was added in order to bring the total volume to 50 mls. This single centrifugation tube (per subject) was spun again at 400G for ten minutes. At the completion of this spin the supernatant was discarded and 5 mls FACS buffer was added to the pelleted PBMCs. These cells were resuspended and 10 μ L was added to an Eppendorf microtube pre-loaded with 90 μ L Trypan Blue Solution 0.4% (ThermoFisher Scientific, Waltham, MA) for cell counting using a Neubauer haemocytometer.

Following cell counting for viability, FACS buffer was added to the isolated PBMCs to bring the total volume to 50 mls. The cells were spun at 400G for ten minutes and the supernatant discarded. The isolated PBMCs were then resuspended in a pre-calculated volume of 10% FCS and RPMI-1640 Medium (Sigma-Aldrich, Macquarie Park, Australia) in order to yield 1×10^7 PBMCs per 500 μ L. Prior to freezing, 500 μ L of 20% dimethyl sulfoxide (DMSO), 10% FCS and RPMI-1640 was aliquoted into 1 ml Nunc CryoTubes (Sigma-Aldrich, Macquarie Park, Australia) which were placed on ice. Subsequently, 500 μ L of PBMCs suspended in RPMI-1640 were added to preloaded CryoTubes to yield 1×10^7 PBMCs in 1 ml 10% DMSO with 10% FCS in RPMI-1640. The CryoTubes were then transferred to a Mr. Frosty Freezing Container (Sigma-Aldrich, Macquarie Park,

Australia) loaded with 500 mls 100% isopropyl alcohol for controlled freezing at -80C prior to transfer to liquid nitrogen for long term storage. The isopropyl alcohol used for controlled freezing was exchanged after every fifth freeze cycle.

On average, 100 mls whole blood was collected from each patient and control subject at each time point. From that volume, approximately 1×10^8 PBMCs were isolated and stored in liquid nitrogen. In addition to an analysis of PBMC expression of MERTK, approximately 5 mls of blood from each patient and control subject was aliquoted for DNA isolation. The *MERTK* polymorphism at rs7422195 was also ascertained.

2.11.2 Flow Cytometric Analysis

Nunc CryoTubes were retrieved from liquid nitrogen storage and transferred on dry ice. Paired tubes from patient samples taken at the time of study entry and three months after first collection were rapidly thawed in a water bath at 37°C until a slushy consistency was achieved. The 10% DMSO with 10% FCS in RPMI-1640 solution containing the PBMCs was then rapidly transferred to a 15 ml centrifugation tube pre-loaded with 10 mls 10% FCS in RPMI-1640 Medium, stored on water ice. After all samples for analysis were transferred to 15 ml centrifugation tubes, they were spun at 400G for ten minutes with the brake on.

After centrifugation, the supernatant was aspirated by glass pipette and 500 uL of FACS buffer was added to the pelleted PBMCs. For assessment of cell viability, 10 uL of resuspended PBMCs were added to 90 uL Trypan Blue Solution 0.4% and cells were counted with a Neubauer haemocytometer.

Based on the cell count for each sample, a calculated volume of PBMCs in 10% FCS in RPMI-1640 solution was transferred to Eppendorf microtubes to yield 1×10^6 PBMCs per tube for staining for flow cytometric analysis. The sample with the greatest number of viable cells was used for flow cytometry compensation. After transfer of all samples to Eppendorf microtubes, the samples were spun at 400G for five minutes. The

supernatant was discarded by manual pipetting. Subsequently, 50 uL of the requisite flow cytometry antibodies at pre-calculated dilutions were then added to each sample. The cells were resuspended and set aside to incubate at 4C for thirty minutes. After incubation, 300 uL FACS buffer was added to each Eppendorf microtube and the specimens were spun for a further five minutes at 400G to remove unbound antibodies. The supernatant was discarded by manual pipetting and 100 uL of FACS buffer was added to each sample to resuspend the PBMCs. Prelabelled FACS tubes in the order of the FACS analysis to be performed were filled with 200 uL FACS buffer. The PBMCs were then passed through a fine mesh filter into the corresponding FACS tube by manual pipetting.

In order to assess MERTK expression on the surface of monocytes, PBMCs from MS patients and healthy controls were identified using antibodies directed against the markers CD14-PE (TUK4) and CD16-FITC (VEP13)(both from Miltenyi Biotec, Bergisch Gladbach, Germany). Cell surface MERTK protein was detected using human Mer APC-conjugated antibody (Clone #125518 (R&D Systems, Minneapolis, MN)) and compared with the appropriate isotype control (APC-conjugated Mouse IgG1, IS5-21F5 (Miltenyi Biotec, Bergisch Gladbach, Germany)). Classically activated monocytes were identified by high levels of CD14 (a co-receptor for Toll-like receptor 4, mediating lipopolysaccharide signalling) and low levels of CD16 (Fc gamma receptor IIIa). Non-classical monocytes were identified by low levels of CD14 and high levels of CD16.¹⁴⁹ PBMCs were then analysed on a CytoFlex S Analyzer (Beckman Coulter, Pasadena, CA). Median fluorescence intensity and mean proportion $\pm$ SEM calculations were performed using either FlowJo 9 (FlowJo, Ashland, OR) or FSC Express 6/7 Research Edition (De Novo Software, Glendale, CA).

2.11.3 Restriction Fragment Length Polymorphism Genotyping

Human DNA was isolated from 5 – 10 mls whole blood, collected contemporaneously with samples for PBMC isolation. DNA purification was performed using either the illustra™ Nucleon Genomic DNA Isolation Kit (RPN8502, GE Healthcare,

Buckinghamshire, UK) or the QIAamp DNA Mini Kit (QIAGEN NV, Venlo, The Netherlands) in accordance with the manufacturers' instructions. Concentration and purity of DNA was quantified using a Nanodrop spectrophotometer (Biolab Scientific, Mumbai, India).

To amplify human specimens, PCR was used in the reaction listed in Table 2.7. PCR products were run on a 1.5% (w/v) agarose/TAE gel (ThermoFisher Scientific, Waltham, MA) with 1:10,000 (v/v) SYBR Safe cyanine dye ((ThermoFisher Scientific, Waltham, MA), visualised under ultraviolet illumination. Forward and reverse primers for *MERTK* rs7422195 were designed using the University of Waterloo restriction analysis tool (<http://watcut.uwaterloo.ca/template.php>).

Table 2.6: Human Polymerase Chain Reaction

Process	Reagents	PCR Conditions
Genomic DNA Amplification	1x PCR buffer 1.5 nM MgCl ₂ 200 nM dNTPs 0.8µM Forward Primer CACCCACATCCTGCTGATTA 0.8µM Reverse Primer ATCCTTCCATTGCTTGCTG 1U Taq Polymerase Total volume: 25µl	94°C - 3 min 98°C - 30 sec 60°C - 30 sec 72°C - 1 min 72°C - 3 mins } x30

Following DNA amplification, restriction digestion was used to identify *MERTK* genotypes at rs7422195 with the reaction set out in Table 2.8 using CutSmart buffer and Ava (I) enzyme (New England BioLabs, Ipswich, MA).

Table 2.7: Restriction Digestion

Process	Reagents	Thermal Conditions
<i>MERTK</i> rs7422195 identification	Ava (I) Enzyme 0.5 µL CutSmart Buffer 2 µL H ₂ O 12.5 µL PCR Product 5 µL Total volume: 20µl	37°C - 2 hours 80°C - 20 mins

2.11.4 *MERTK* Expression Analysis in Monocytes Using Quantitative PCR

Total RNA was isolated from purified monocytes using the Qiagen RNeasy minikit (Qiagen NV, Venlo, The Netherlands) according to the manufacturer's instructions. RNA was then reverse transcribed into cDNA using a Taqman Reverse Transcription kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA) using the steps set out in Table 2.9. Negative controls were also prepared by excluding reverse transcriptase to assess for potential genomic DNA contamination. Samples were run in a thermal cycler under the following conditions: 25C for 10 minutes; 48C for 30 minutes; and 95C for 5 minutes.

Table 2.8: Reverse Transcription Reactions

Reagent	Reverse Transcription	Negative Control
Taqman RT Buffer	1X	1X
MgCl ₂	5.5 mM	5.5 mM
dNTPs	2 mM	2 mM
Random Hexamers	40U	40U
RNase Inhibitor	125U	125U
Multiscribe RT	16U	-

Quantitative analysis was performed on a ViiA 7 Real Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR Green master mix (ThermoFisher Scientific, Waltham, MA). Thermal cycling conditions are set out in Table 2.10. A dissociation step was included to ensure PCR product specificity.

Table 2.9: *MERTK* Quantitative PCR Reactions

Machine	Reagents	Thermal Cycler Conditions
ViiA 7	1x SYBR Green Master Mix 0.8µm Forward Primer CTGCCGAGAAGTGGGAAGAG 0.8µm Reverse Primer CCCTGGTAAAGGCCCTGAAA cDNA: 5µl Total volume: 25µl	50°C - 2 min 95°C - 10 min 60°C - 1 min } x45

2.12 Quantification of Human MHC-II and MERTK by Histochemistry

Photomicrographs of chronic active demyelinating plaques from autopsy specimens of patients with secondary progressive MS were kindly provided by Assistant Professor Ranjan Dutta, Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University. The images were opened in Adobe Photoshop CC 2018 (Adobe Systems, San Jose, CA) and a pre-defined area of interest (500 x 500 pixels) was identified at the peri-plaque border. All cells of interest in the outlined area were counted. All results are expressed as cells/mm² ±SEM.

2.13 Statistical Analysis – Human Experiments

All statistical tests were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). A *p*-value of <0.05 was considered statistically significant. For flow cytometric and histochemical analyses, the Student's T test was used to compare groups. Results are expressed as mean proportion ±SEM. Simple linear regression was used to analyse the relationship between MHC-II and MERTK expression in histochemical sections. For lesional expression of MERTK among rs7422195 genotypes one-way ANOVA analysis with Tukey's multiple comparisons test was used to determine statistical significance.

2.14 Human Research Ethics Approval.

Recruitment of subjects and collection of tissue and DNA was approved by the Melbourne Health Human Research Ethics Committee (Project number: 2013.111). The Human Research Ethics Committee which provided approval for this research is guided by national standards as outlined in the National Statement on Ethical Conduct in Human Research (<https://www.nhmrc.gov.au/guidelines-publications/e72>) issued by the National Health and Medical Research Council (Australia). All cases and controls provided written consent for the use and storage of cellular and DNA samples.

CHAPTER 3: RESULTS

MURINE EXPRESSION OF *MERTK* IN HEALTH AND DEMYELINATING DISEASE

3.1 Introduction

It is well established that GAS6, a cognate ligand of all three TAM receptors, can modify the outcomes of inflammatory^{140, 150} and toxic^{103, 105, 106} mouse models of demyelination. As outlined in Chapter 1, previous investigations have identified disease- and tissue-specific roles for each TAM receptor in health and disease.¹⁵¹ However, the individual contributions of each member of the TAM family to inflammatory demyelination is yet to be fully described. While the role of AXL in modifying the outcomes of experimental models of MS has been previously described,¹⁵² the relative contributions of TYRO3 and MERTK require further elucidation.

As previously described in Chapter 1, *MERTK* is a known risk allele for susceptibility to MS.¹⁰¹ More detailed assessments of this association demonstrate independent signals from common and low frequency variants of *MERTK*. Intriguingly, one high expressing variant is associated with either increased or decreased risk of MS susceptibility, depending on HLA haplotype – specifically upon *HLA-DRB1*15:01* status.¹⁵³ The *HLA-DRB1*15* allele has long been associated with the development of MS in populations from northern Europe and is the strongest risk gene for the development of MS.²⁴

As a result of these findings, I hypothesised that MERTK contributed, at least in part, to the altered outcomes observed in mouse models of demyelination after GAS6 manipulation. In order to test this hypothesis, a conditional knock-out (cKO) mouse was developed to examine the role of MERTK in inflammatory and toxic demyelination. A model based upon a germline mutation in MERTK has already been described. These animals express a truncated version of MERTK in which the intracellular tyrosine kinase domain is absent.¹⁵⁴ However, as these animals experience impaired clearance of apoptotic cells from birth there are rapid phenotypic changes that render this model

less useful. In particular, the animals develop a progressive autoimmune phenotype, presumably due to excess burden of auto-antigen.¹³⁸ As a result, these animals are not suited to assessment of immune responses, for example following EAE induction. In order to overcome the early establishment of an abnormal phenotype, *Mertk* was targeted using the Cre-loxP conditional deletion method.

In particular, CD11c⁺ cells were targeted for discrete *Mertk* deletion with the Cre-loxP model. CD11c⁺ cells include peripheral dendritic cells but also a proportion of microglia, cells that among other functions act as tissue-resident macrophages in the CNS.⁶⁹ Among innate immune cells, dendritic cells serve as professional antigen presenting cells and a link between the innate and adaptive immune systems. Dendritic cells express high levels of MERTK in response to pro-inflammatory signals. By this mechanism, dendritic cells provide anti-inflammatory autoregulation, modulating immune responses.¹³⁹ In addition, dendritic cells regulate T cell activation directly via MERTK-signalling¹⁵⁵ and indirectly through competition for the Pros1 ligand, which would otherwise interact with MERTK expressed by T cells in a pro-inflammatory manner.¹³²

In order to determine whether conditional deletion of *Mertk* altered immune development and maturation, immune cell populations were assessed in unchallenged WT and MERTK deficient animals. In addition, TAM receptor expression at the RNA level in the spinal cord and by dendritic cells was assessed following EAE induction. As noted in Chapter 2, this work was performed in animals from a mixed C57BL/6 and 129/SvEv background. These data were generated by Dr. Gerry Ma¹⁵⁶ and are reproduced here in order to assist with the analyses of EAE outcomes that follow in this and subsequent chapters.

3.2 Confirmation of Conditional Knock-out of *Mertk* in CD11c⁺ Cells by Cre-loxP Recombination.

The construction of the *Mertk* floxed mouse and the breeding strategy employed to prepare animals for experimentation are set out in Chapter 2. Conditional deletion of MERTK at the protein level was confirmed in CD11b⁺ CD11c⁺ cells by flow cytometric analysis. Among dendritic cells, CD11b⁺ CD11c⁺ cells were considered most likely to directly influence EAE outcomes.⁸⁶ The gating strategy to identify these cells by flow cytometric analysis is set out in **Figure 3.1a**. MERTK events were virtually absent from these CD11c⁺ cells isolated from the spleens of CD11c-Cre^{+/+}/*Mertk*^{fl/fl} animals compared to Cre^{+/+} controls (1712 ± 320 events (SEM), *p*=0.002). A statistically significant reduction in MERTK protein expression was seen in CD11c-Cre^{+/+}/*Mertk*^{fl/wt} animals compared to CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls (534 ± 191 events (SEM), *p*=0.01). One-way ANOVA among the means of the three cohorts was also highly significant (*p*<0.0001) (**Fig 3.1b**).

The extent of recombination was determined at the RNA level by quantitative PCR using primers in which an amplified band (Δ *Mertk*) is generated selectively in response to genetic recombination in the floxed *Mertk* mice. The primer design for Δ *Mertk* is set out in Chapter 2. To assess the level of deletion, CD11c⁺ cells were isolated from CD11c-Cre^{+/+}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/+}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice. Quantitative PCR confirmed highly efficient deletion of *Mertk* from CD11c⁺ cells in CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice compared to controls (**Fig. 3.1c**).

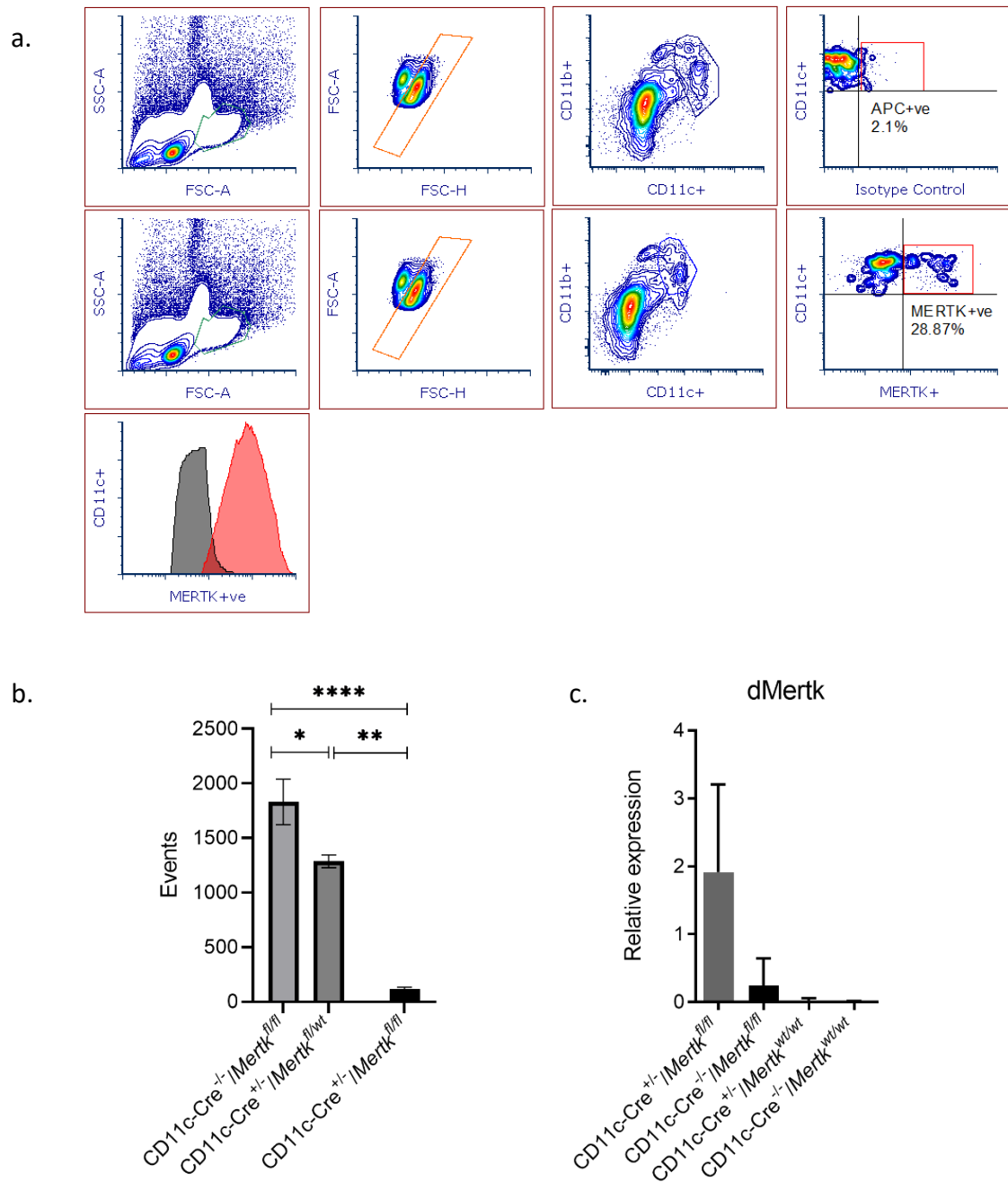


Figure 3.1. Conditional Deletion of *Mertk* from CD11c⁺ Cells in CD11c-Cre^{+/+}/*Mertk*^{fl/fl} Mice. **a.** Gating strategy for identification of CD11b⁺ CD11c⁺ dendritic cells; **b.** Number of MERTK positive events by flow cytometric analysis in CD11b⁺ CD11c⁺ cells from spleens of CD11c-Cre^{+/+}/*Mertk*^{fl/fl}, CD11c-Cre^{+/+}/*Mertk*^{fl/wt} and CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice, n=4-12 per genotype; **c.** Quantitative PCR analysis of Δ *Mertk* expression in splenic CD11c⁺ cells from CD11c-Cre^{+/+}/*Mertk*^{fl/fl}, CD11c-Cre^{+/+}/*Mertk*^{fl/wt}, CD11c-Cre^{+/+}/*Mertk*^{wt/wt} and CD11c-Cre^{+/+}/*Mertk*^{wt/wt} mice. n=2-4 per genotype. Student's T-test, ***p*=0.002, **p*=0.01. One-way ANOVA with Sidak's multiple comparisons, *p*<0.0001.

3.3 Conditional Deletion of *Mertk* From CD11c⁺ Cells Does Not Affect Immune Cell Subtypes Under Physiological Conditions.

To determine if *Mertk* deletion from CD11c⁺ cells altered immune development, the proportions of immune cell subtypes in the bone marrow, spleen and lymph nodes of CD11c-Cre^{+/+}/*Mertk*^{fl/fl} and control genotypes were assessed by flow cytometric analysis. No differences in the proportions of: CD4⁺ T cells; CD8⁺ T cells; CD19⁺ B cells; CD11c⁺ dendritic cells; CD11b⁺Ly6C^{lo} resident monocytes; CD11b⁺Ly6C^{hi} inflammatory blood monocytes; CD11b⁺Gr-1⁺ granulocytes; CD11c⁺CD11b⁻B220⁺ plasmacytoid dendritic cells; or CD11c⁺CD11b⁺B220⁻ myeloid dendritic cells were identified between genotypes (**Fig. 3.2a-k**).

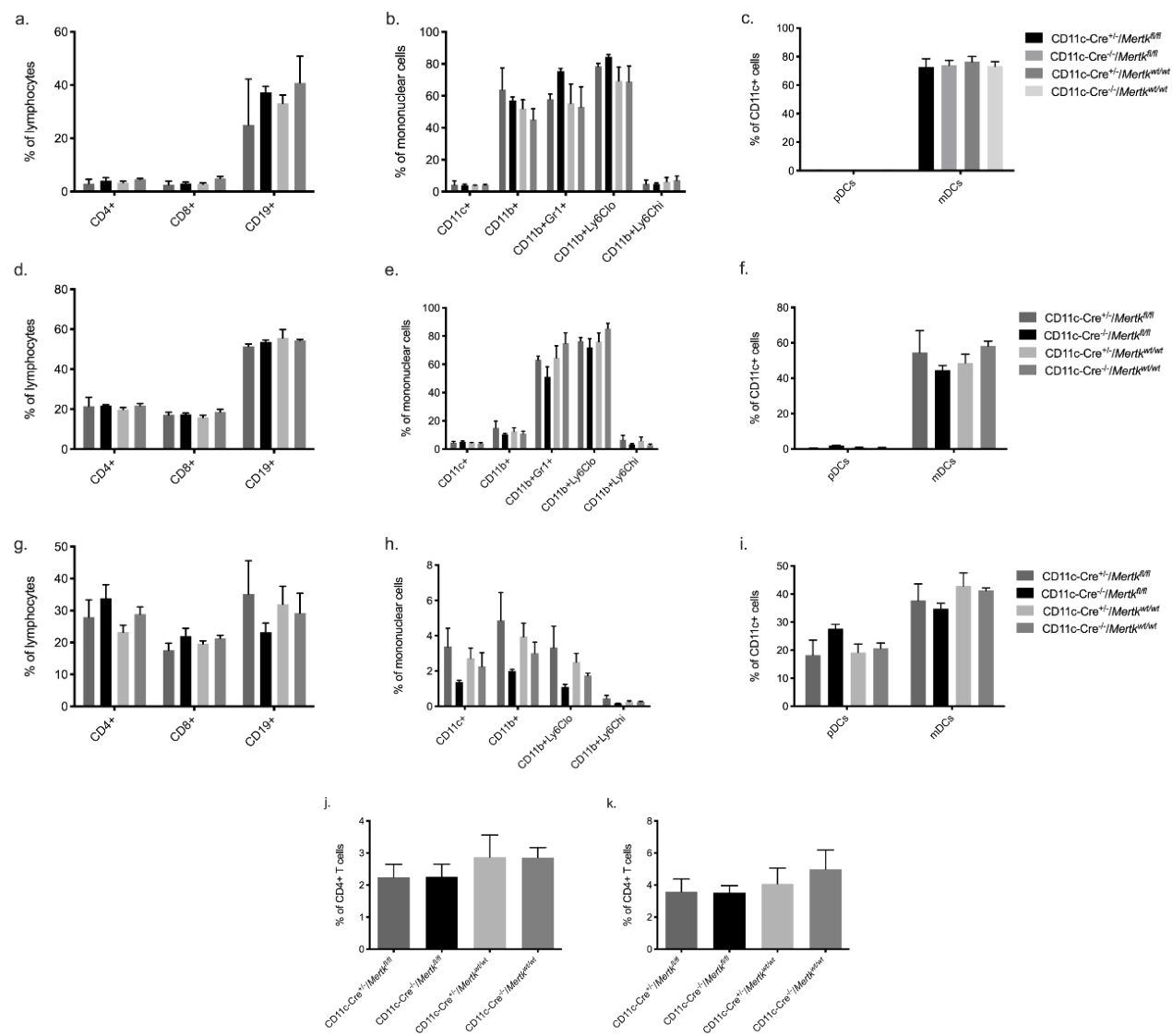


Figure 3.2. Conditional deletion of *Mertk* from CD11c⁺ cells does not alter resting immune cell proportions. **a-c.** Quantification of immune cell subtypes in the bone marrow of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice; **d-f.** Quantification of immune cell subtypes in the spleen of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice; **g-i.** Quantification of immune cell subtypes in the lymph nodes of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice; **j.** Quantification of CD4+CD25hi regulatory T cells in the spleen of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice; **k.** Quantification of CD4+CD25hi regulatory T cells in the lymph nodes of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{-/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{wt/wt} and CD11c-Cre^{-/-}/*Mertk*^{wt/wt} mice. *n*=2-3 per genotype.

3.4 Expression of *Tyro3*, *Mertk* and *Axl* Changes in the Lumbar Spinal Cord During EAE in Wild-Type C57BL/6 Mice.

In order to assess TAM receptor expression in affected tissues during EAE, the lumbar expansion of the spinal cord was harvested at time points correlating with the effector phase of immune-mediated demyelination (8 days post-induction (dpi)) or at disease peak (15-21 dpi) with unchallenged 8-12 week old adult mice used as controls. Quantitative PCR was used to measure levels of TAM receptor gene expression along with the TAM ligand genes, *Pros1* and *Gas6*. All TAM genes demonstrated differential regulation during EAE. Two expression patterns were observed. Upregulation of *Axl*, *Mertk* and *Pros1* gene expression was noted with EAE progression (**Fig. 3.3a**), while downregulation of *Tyro3* and *Gas6* was identified at 15 dpi, remaining low until 21 dpi (**Fig 3.3b**). In particular, expression of *Mertk* was increased at 15 and 21 dpi, peaking at 3.4-fold at 21 dpi ($p<0.001$) while *Tyro3* was decreased at 15 and 21 dpi (0.4 fold for both time points, $p<0.001$). When stratified by sex, no statistically significant difference in TAM receptor or ligand gene expression was identified (**Fig. 3.3c-l**), with the exception of *Mertk*, where a significantly increased level of expression was noted in male mice experiencing clinical scores between two and three (**Fig. 3.3j**).

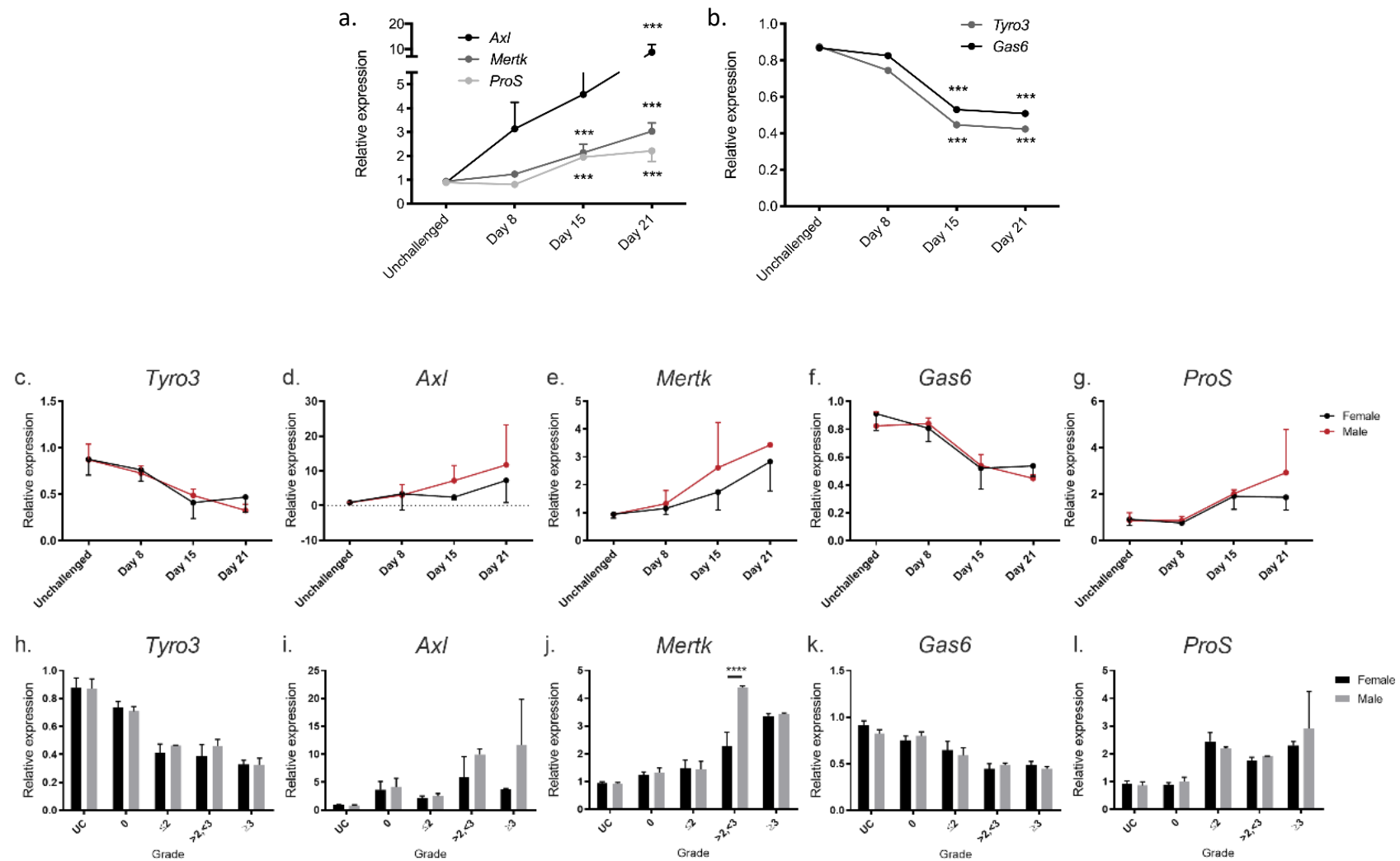


Figure 3.3. TAM receptor and ligand genes are differentially regulated in the lumbar spinal cord during EAE. **a.** Expression of the TAM receptor genes *Axl* and *Mertk*, and the TAM ligand gene *ProS* are upregulated in the spinal cord during EAE; **b.** The TAM receptor gene *Tyro3* and the TAM ligand gene *GAS6* are downregulated in the spinal cord and decrease with EAE disease progression. One-way ANOVA, Tukey's multiple comparisons test, *** $p < 0.0001$, $n = 7-18$ per time point; **c-g.** Expression of the TAM genes in the EAE spinal cord stratified by sex; **h-l.** Expression of the TAM genes in the EAE spinal cord stratified by grade. UC: unchallenged. Two-way ANOVA, Sidak's multiple comparisons, **** $p < 0.0001$, $n = 2-7$ per time point.

3.5 Dendritic Cell Expression of *Tyro3*, *Mertk* and *Axl* Changes During EAE in Wild-Type Mice.

To determine whether *Mertk* expression changed in CD11c⁺ cells in wild-type mice following EAE induction, CD11c⁺ cells were isolated by fluorescent-activated cell sorting from the spleens of wild-type mice following EAE induction. The level of gene expression of *Tyro3* and *Axl*, along with the TAM ligand genes *Gas6* and *Pros1* was also assessed.

In dendritic cells, expression of all TAM receptor genes and the gene for the ligand ProS was altered during EAE. Expression of *Tyro3* (**Fig. 3.4a**) and *Pros1* (**Fig. 3.4e**) were both transiently increased at 8 dpi (relative to unchallenged, 3.16 ± 0.56 (SEM) fold, and 1.52 ± 0.18 fold, respectively; $p < 0.05$) while *Axl* and *Mertk* levels were elevated at 8 and 15 dpi (2.67 ± 0.54 and 3.02 ± 0.50 , respectively at 15 dpi; $p < 0.05$)(**Fig. 3.4b,c**). One-way ANOVA revealed a difference in the means for each time point for both *Axl* and *Mertk* ($p < 0.05$). No change in *Gas6* gene expression was observed (**Fig. 3.4d**). No sex-specific differences in TAM receptor gene expression were identified in dendritic cells (not shown).

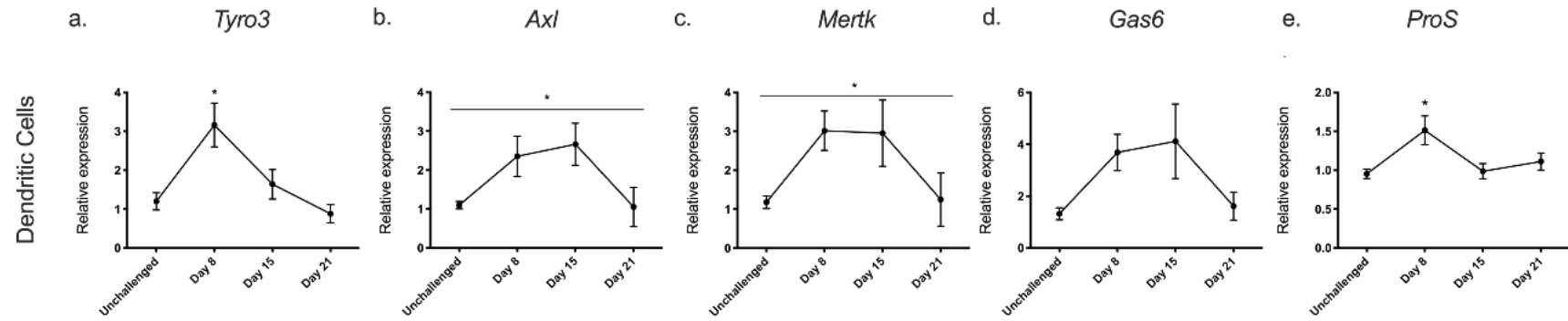


Figure 3.4. TAM receptor and ligand genes are differentially regulated in dendritic cells during EAE. a-e. Expression of the TAM receptor genes in dendritic cells of EAE mice. One-way ANOVA, Tukey's multiple comparison test, * $p < 0.05$, $n = 7-12$ per time point.

3.6 Conditional Deletion of *Mertk* in CD11c^{+ve} Cells Results in Decreased MERTK Levels Following EAE Induction.

MERTK expression at the protein level was then assessed in CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{fl/wt} and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice following EAE induction. This experiment was performed in order to assess whether heterozygous deletion of *Mertk* in CD11c^{+ve} cells could be overcome in the highly inflammatory environment induced during EAE. Mice were assessed at day 18, in order to perform protein analysis at disease peak. CD11b^{+ve}CD11c^{+ve} cells from the spleens of CD11c-Cre^{+/-}/*Mertk*^{fl/fl}, CD11c-Cre^{+/-}/*Mertk*^{fl/wt} and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice were isolated and examined by flow cytometric analysis. The gating strategy noted in **Figure 3.1a** was applied.

No significant difference in MERTK levels were noted among genotypes based on median fluorescence intensity (MFI)(**Fig. 3.5a**). I hypothesised that a small number of very bright events were obscuring a significant difference in the proportion of MERTK^{+ve} cells in these samples. This hypothesis was supported by analysis of MERTK^{+ve} events, where inter-genotype differences in MERTK levels were observed. As expected, *Mertk* homozygous deleted mice demonstrated extremely low levels of MERTK, while heterozygous deleted mice demonstrated significantly lower levels of MERTK compared to CD11-Cre^{-/-} controls (688 ± 108 events (SEM), $p=0.0001$) (**Fig. 3.5b**). This indicates that EAE induction and the subsequent development of inflammatory immune responses is not sufficient to overcome the effect of heterozygous gene deletion in CD11c^{+ve} cells. Sex was not a determinant of MERTK levels following conditional deletion, with reduction in MERTK levels consistent in male (538 ± 110 event (SEM), $p=0.0006$) and female (759 ± 152 events (SEM), $p<0.0001$) mice (Fig 3.5c). No statistically significant sex-based differences in MFI or MERTK^{+ve} events were identified within genotypes (**Fig. 3.5d-i**).

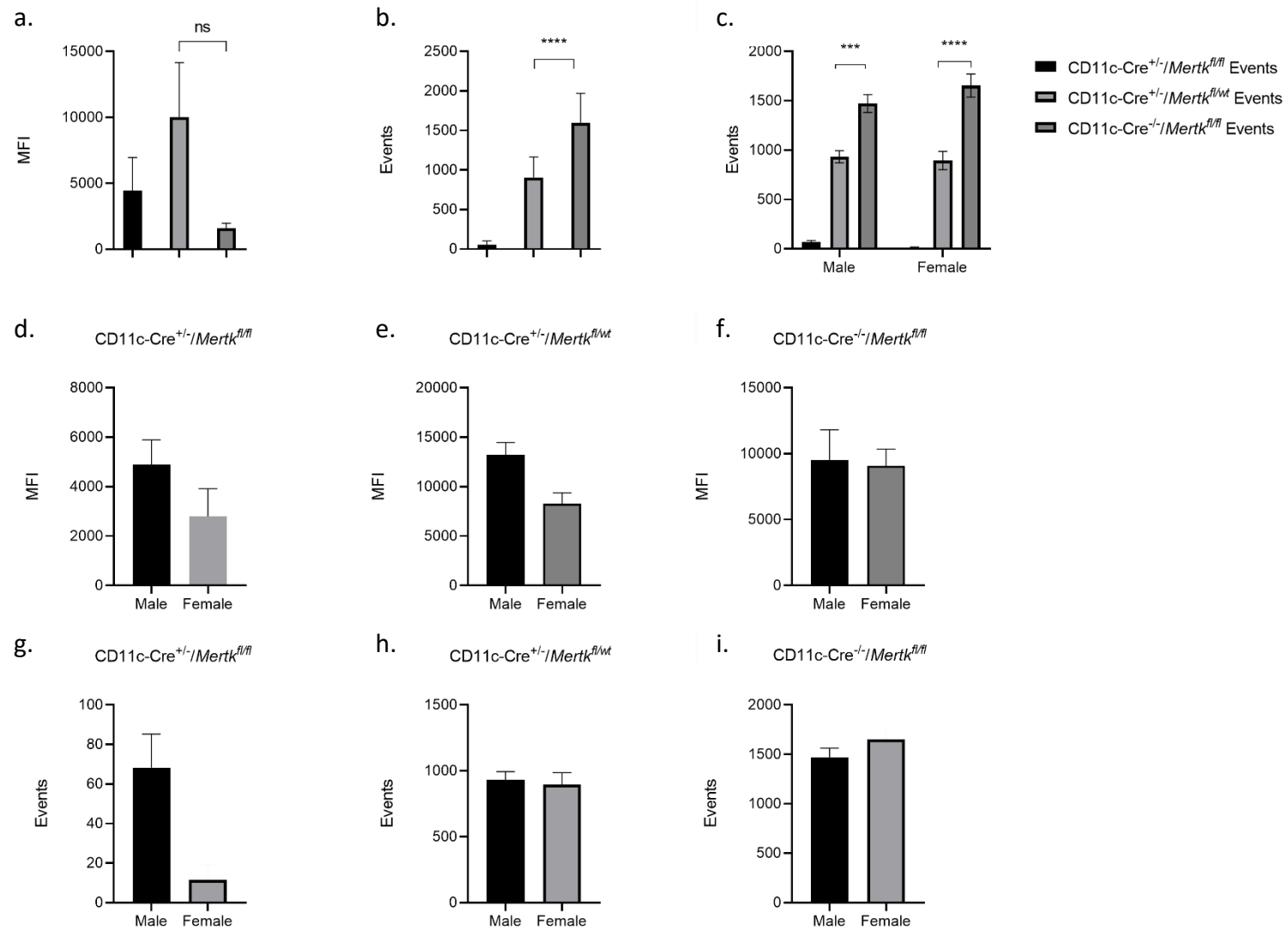
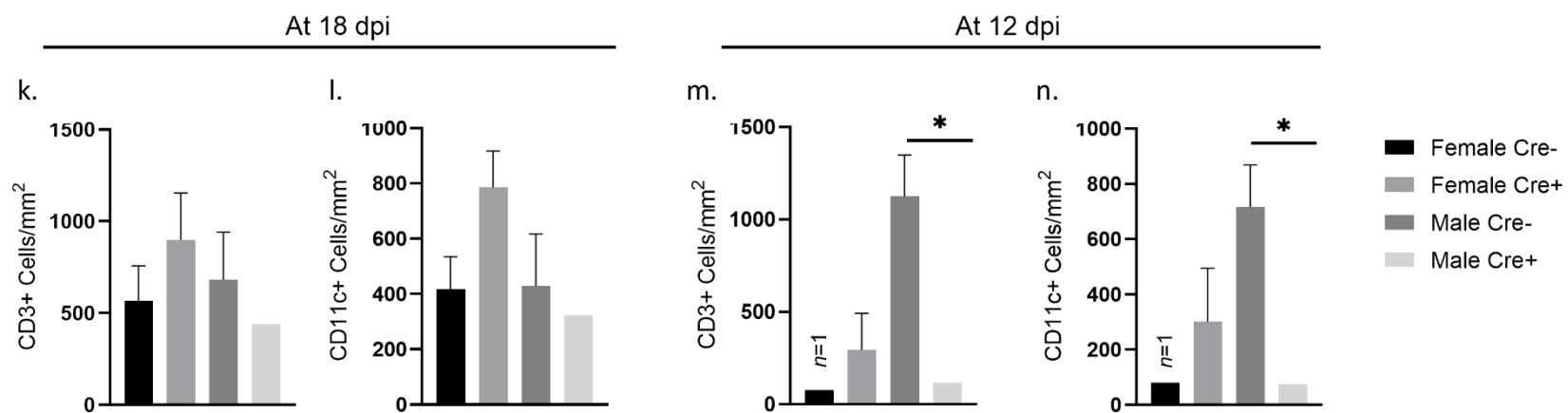
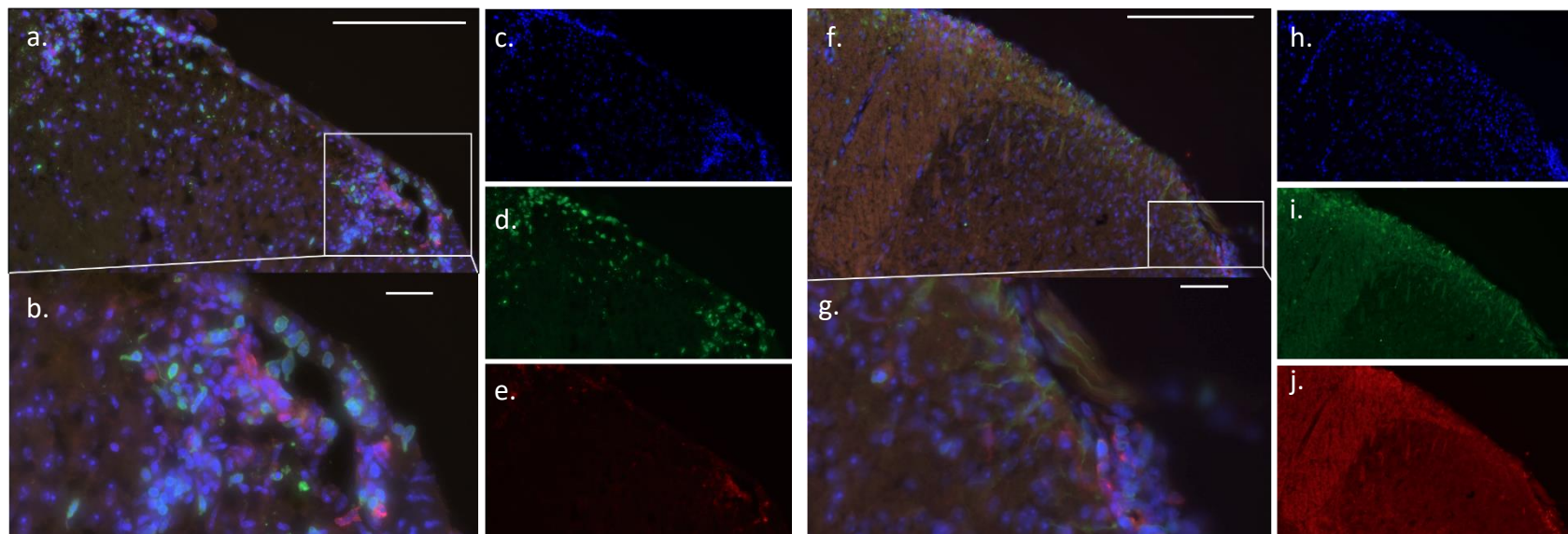


Figure 3.5. Conditional Deletion of *Mertk* in CD11c⁺ Cells Results in Decreased MERTK Levels Following EAE Induction. **a.** Flow cytometric analysis of MERTK expression in CD11b⁺ CD11c⁺ splenocytes by median fluorescence intensity (MFI); **b.** Flow cytometric analysis of MERTK expression in CD11b⁺ CD11c⁺ splenocytes by number of events, indicating near complete absence of expression in CD11c-Cre^{+/+}/*Mertk*^{fl/fl}, with dose reduction in CD11c-Cre^{+/+}/*Mertk*^{fl/wt} mice compared to CD11c-Cre^{+/+}/*Mertk*^{fl/fl} controls (Student's T-test, *****p* < 0.0001); **c.** Flow cytometric analysis of *Mertk* expression in CD11b⁺ CD11c⁺ splenocytes by number of events stratified by sex; significant difference between male CD11c-Cre^{+/+}/*Mertk*^{fl/wt} and CD11c-Cre^{+/+}/*Mertk*^{fl/fl} controls (Student's T-test, ****p* = 0.0006) and significant difference between female CD11c-Cre^{+/+}/*Mertk*^{fl/wt} and CD11c-Cre^{+/+}/*Mertk*^{fl/fl} controls (Student's T-test, *****p* < 0.0001); **d-i.** No sex-specific difference MERTK expression by number of events in CD11b⁺ CD11c⁺ splenocytes within CD11c-Cre^{+/+}/*Mertk*^{fl/fl}, CD11c-Cre^{+/+}/*Mertk*^{fl/wt} or CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice. *n* = 4-8 per sex per genotype.

3.7 Conditional Deletion of *Mertk* In CD11c⁺ Cells Does Not Alter Inflammatory Spinal Cord Infiltrates Following EAE Induction.

In order to assess levels of immune activation in EAE-treated mice, the number of CD3⁺ T cells and CD11c⁺ dendritic cells were quantified within the dorsal column of the lumbar spinal cord of CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls collected at 18 dpi. This time point allowed for quantification of immune infiltrates at disease peak. A single cohort of CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls were killed at 12 dpi in order to quantify immune infiltrates during the effector stage of EAE.

No difference in cells counts of either CD3⁺ or CD11c⁺ cells were identified in CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls killed at 18 dpi (**Fig. 3.6k,l**). A statistically significant difference in immune cell infiltrate in male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice killed during the effector phase of EAE was observed compared to male CD11c-Cre^{+/+}/*Mertk*^{fl/fl} homozygous deleted animals. Differences were seen in both CD3⁺ (1000 ± 300 cells/mm² (SEM), *p*=0.04) (**Fig. 3.6m**) and CD11c⁺ cells (642 ± 201 cells/mm² (SEM), *p*=0.049) (**Fig. 3.5n**). Within-grade analysis identified a relationship between clinical severity index and degree of inflammatory infiltrate in samples collected at 18 dpi (**Fig. 3.5o,p**) but not at 12 dpi (**Fig. 3.5q,r**).



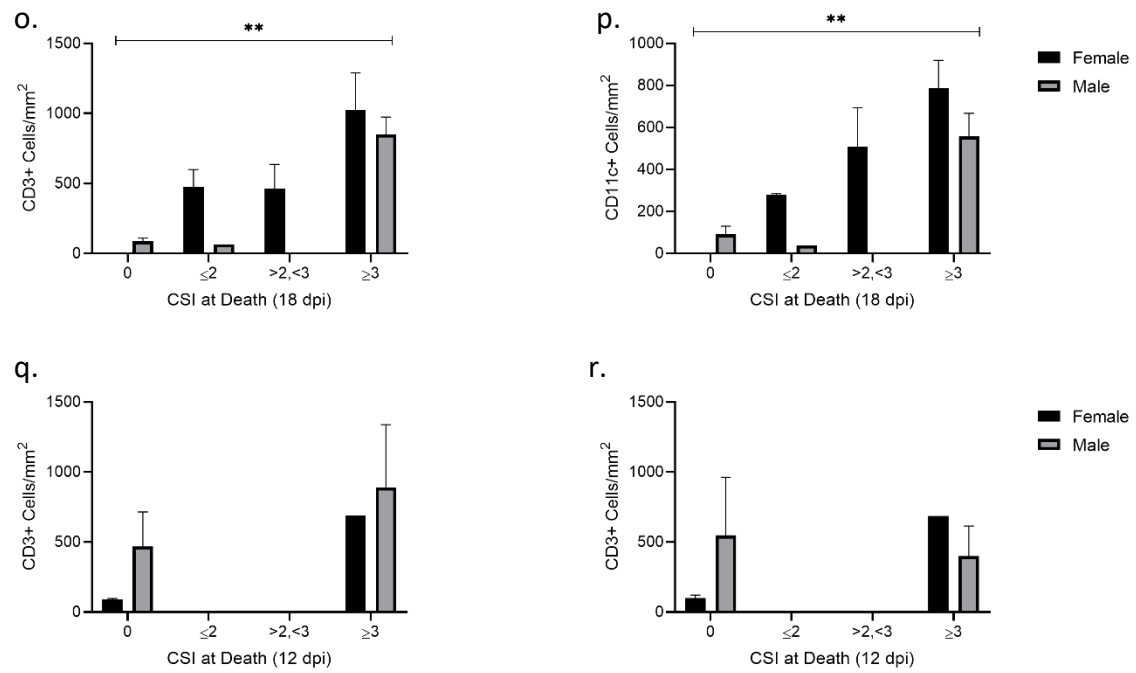


Figure 3.6. Conditional deletion of *Mertk* in CD11c⁺ cells does not alter inflammatory spinal cord infiltrates following EAE induction. a-e. Representative micrograph of mouse dorsal column in lumbar spine from CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mouse stained for DAPI (c), CD3 (d) and CD11c (e); f-j. Representative micrograph of mouse dorsal column in lumbar spine from CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mouse stained for DAPI (h), CD3 (i) and CD11c (j); k-l. Cell counts per mm² of CD3⁺ and CD11c⁺ cells in the lumbar spinal cord of CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls stratified by sex; animals killed at 18 dpi; n=4-5 per sex per genotype; m-n. Significant increase in cell counts per mm² of CD3⁺ and CD11c⁺ cells in the lumbar spinal cord of CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice compared to CD11c-Cre^{+/+}/*Mertk*^{fl/fl} littermates; animals killed at 12 dpi to examine infiltrates in effector stage of EAE (Student's T-test, (*p=0.044 for CD3)(*p=0.049 for CD11c)); no difference in female CD11c-Cre^{+/+}/*Mertk*^{fl/fl} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls; n=2-3 per sex genotype except female CD11c-Cre^{-/-}/*Mertk*^{fl/fl} n=1; o-p. Immune infiltrates of CD3⁺ and CD11c⁺ cells stratified by EAE clinical severity index at 18 dpi. Two-way ANOVA, significant for row effect (CSI) p<0.0015 for both cell types, Sidak's multiple comparison test; q-r. Immune infiltrates of CD3⁺ and CD11c⁺ cells stratified by EAE clinical severity index at 12 dpi. Two-way ANOVA, Sidak's multiple comparison test. Scale bars: a, f: 100 μm, b-g: 20 μm.

3.8 Discussion

Quantitative PCR analysis confirmed efficient deletion of *Mertk* from CD11c⁺ cells in cKO mice by excision of exon 2 from the *Mertk* gene, validating the use of the Cre-loxP model of conditional deletion. In addition, flow cytometric analysis did not demonstrate any differences in innate or adaptive immune cell populations under physiological conditions. This suggests the MERTK expressed by CD11c⁺ cells is not required for normal immune cell development.

Following EAE induction, all TAM genes demonstrated differential regulation in the spinal cords of affected mice. Upregulation of *Axl*, *Mertk* and *Pros1* was noted with EAE progression, while downregulation of *Tyro3* and *Gas6* was identified at 15 dpi, remaining low until 21 dpi. A similar pattern was noted in the study by Binder et. al. (2008) where *Axl*, *Mertk* and *Gas6* were upregulated following cuprizone intoxication, along with downregulation of *Tyro3* in the corpus callosum.¹⁰⁵ Gruber et. al. (2014) also noted increased *Axl* and *Gas6* levels 8 days after onset of EAE symptoms (~17 dpi) while *Mertk* expression was increased at days 4 (~13 dpi) and 8 after symptom onset. However, in that study no differences in *Tyro3* or *Pros1* levels with EAE progression were noted.¹⁰⁴ While EAE and cuprizone models cannot be directly compared due to differing mechanisms of demyelination, it does appear that a pattern of *Axl* and *Mertk* upregulation in response to CNS demyelination is established. In addition, there are important technical differences that distinguish the study conducted by Gruber et. al. from the data presented here. Specifically, a different EAE induction method was employed in the Gruber study which results in very rapid onset of symptoms. In addition, mice exposed to EAE in the Gruber study experienced near full recovery by 30 dpi, in distinction to animals in our laboratory, where symptoms persist throughout the disease course, similar to descriptions of EAE in the literature.¹⁵⁷

The sexually dimorphic response of *Mertk* expression following EAE induction in males experiencing relatively severe manifestations of central inflammatory demyelination is intriguing. This finding builds on earlier observations by others in our laboratory that

female C57BL/6 mice express significantly increased levels of *Axl* in peripheral monocytes in the unchallenged state but not during EAE.¹⁵⁶ As noted earlier, phagocytic (M2) macrophages ameliorate the outcomes of inflammatory models of central demyelination¹⁴⁶ and these cells express *Mertk* in response to anti-inflammatory stimuli.¹⁵⁸ Signalling through *Axl* is known to induce immune anergy in dendritic cells¹³⁹ and higher basal levels of this receptor in female mice may account for the sexual dimorphism observed in clinical outcomes, which will be described in a proceeding chapter. Specifically, *Axl*-mediated immune modulation in females early in the pathogenesis of EAE may lead to relatively less aggressive responses to MOG₃₅₋₅₅ stimulation, resulting in improved clinical outcomes and a decreased role for *Mertk* in down-regulating maladaptive immune responses in female animals compared to severely affected males.

EAE induction relies on multiple immune adjuvants to reliably reproduce clinical manifestations of central demyelination. In that context, it is notable that the relative differences in *Mertk* expression were observed under physiological *and* EAE conditions in both CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls. These changes were consistent in male and female mice. While it was expected that homozygous deleted animals would express extremely low levels of MERTK in CD11c^{+ve} cells in all scenarios, it was notable that heterozygous deletion remained evident, despite the generation of a substantial immune response from EAE induction. Accordingly, it appears that dendritic cell expression of MERTK is subject to precise regulatory control that is not overwhelmed by the cytokine storm generated during MOG₃₅₋₅₅ inflammatory demyelination. In addition, in light of this result, I hypothesised that a clinical correlation with the degree of MERTK abrogation might be apparent between genotypes, as reviewed in proceeding chapters.

Despite changes in MERTK expression at the protein level in CD11c^{+ve} cells in keeping with gene dosage, immunohistochemical correlation was not observed following EAE induction. In particular, there were no differences in the degree of inflammatory

infiltrate in the lumbar spinal cord of EAE-treated mice with reference to genotype or sex when analysed at disease peak (18 dpi). However, when EAE was terminated during the effector stage of the experiment (12 dpi) a striking difference in both CD3⁺ and CD11c⁺ counts was noted in male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice. This suggests there was substantially increased immune infiltration in these animals compared to *Mertk*-deficient littermates. However, this significance of this result is tempered by the limited number of animals in this experimental cohort and the absence of a relationship between clinical severity and the degree of inflammatory infiltrate when analysed within grade in animals examined at 12 dpi. Indeed, this result is challenging to synthesise. Setting aside the limited number of animals in these cohorts, it would seem counterintuitive that animals with replete levels of MERTK would demonstrate increased levels of inflammatory infiltrate when the preponderance of the scientific literature has identified a role for MERTK in the down-regulation of inflammatory immune responses. When histological results were examined on a case-by-case basis, high degrees of inflammatory infiltrate were noted with absent clinical disease in some specimens, while other animals experienced severe clinical disease without any evidence of immune infiltrate on histological sections. Although three technical replicates were completed for each specimen at varying geographical points in the lumbar spinal cord, the possibility of sampling error in the histological sections cannot be excluded, which could lead to spurious results.

CHAPTER 4: RESULTS

DELETION OF *MERTK* IN CD11C⁺ CELLS EXACERBATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

4.1 Introduction

Multiple sclerosis is a putative autoimmune condition that results in focal areas of central nervous system demyelination, axonal loss and reactive gliosis. While the importance of adaptive immune cells in relapsing-remitting forms of MS was confirmed by the development of therapeutics successfully targeting these cells, the role of innate immunity in the disease process is not well understood. Whether innate immune cells, in particular, peripheral dendritic cells and macrophages or central nervous system (CNS) microglia are implicated in the neurodegenerative processes of MS remains unknown.

As noted previously, dendritic cells serve as professional antigen presenting cells and a link between the innate and adaptive immune systems. Dendritic cells express high levels of *MERTK* in response to pro-inflammatory signals and provide anti-inflammatory counter regulation, thereby modulating immune responses.¹³⁹ Given their important role in maintaining immune homeostasis, the role of dendritic cell modulation of antigen presentation via *MERTK* signalling was investigated using a novel conditional knock-out mouse using the Cre-loxP deletion system where *Mertk* expression in CD11c⁺ dendritic cells is abrogated. Groups of animals deficient in *MERTK* in CD11c⁺ cells were induced with EAE and assessed at various time points in the disease course in order to perform RNA, protein and immunofluorescent analysis at disease onset, during disease peak and in recovery. This work aimed to provide a better understanding of how members of the TAM family of tyrosine kinase receptors might be targeted for therapeutic development in the treatment of MS. In particular, a correlation was sought between the sexual dimorphism observed in MS¹⁵⁹ and the sex-specific differences seen in the modulation of mouse models of demyelination by the TAM receptors and their ligands.

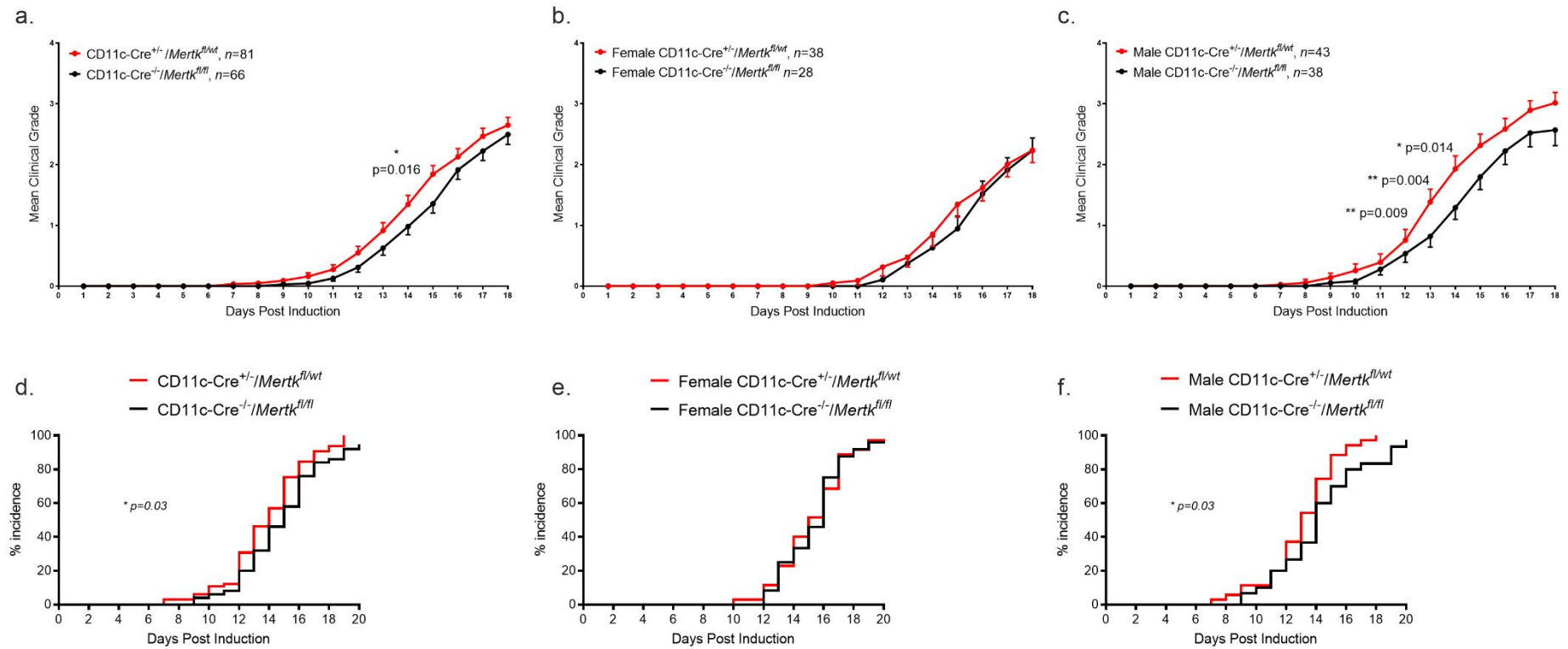
4.2 Heterozygous Deletion of *Mertk* Deficiency in CD11c^{+ve} Cells Results in Exacerbation of EAE in Mice on a C57BL/6 Background.

The role of *Mertk* in the regulation of innate immune responses was interrogated by inducing MOG₃₅₋₅₅ EAE, as described in Chapter 2, in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice. Clinically and statistically significant differences in EAE outcomes were observed in these cohorts. Enhanced disease was observed in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} compared to CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice at 15 dpi. At that time point, the median grade in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice was 2.25 compared to a median grade of 1.0 in CD11c-Cre^{-/-}/*Mertk*^{fl/fl} control mice ($p=0.016$) (**Fig. 4.1a**). Animals with heterozygous deletion of *Mertk* in CD11c^{+ve} cells also demonstrated earlier onset of EAE symptoms. Survival analysis also confirmed greater incidence of EAE in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} animals (Hazard ratio 0.64, 95% CI 0.40 – 0.96, $p=0.03$) (**Fig. 4.1d**).

It was further noted that earlier EAE onset and more severe clinical phenotype were predominantly experienced by male mice. When data were stratified by sex, striking differences in disease onset and severity were identified. There was no difference in EAE severity among female mice at any time point throughout the observation period (**Fig. 4.1b**). The entirety of the observed difference in EAE severity was attributable to male mice. Enhanced disease was observed in male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice compared to male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} control mice at days 12, 13 and 14 dpi ($p<0.014$ for all time points) (**Fig. 4.1c**). There was no difference in the timing of EAE onset noted between female CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls (Hazard ratio 1.0, 95% CI 0.55 – 1.91) (**Fig. 4.1e**). Survival analysis confirmed earlier incidence of EAE in male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice compared to male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls (Hazard ratio 0.52, 95% CI 0.29 – 0.93, $p=0.03$) (**Fig. 4.1f**).

In addition, when the CD11c-Cre^{+/-}/*Mertk*^{fl/wt} cohort was stratified by sex, sexually dimorphic responses to EAE induction were observed. Enhanced disease was observed in male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice compared to female CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice at days 10 through 18 dpi ($p<0.008$ for all time points) (**Fig. 4.1g**). At 14 dpi male CD11c-

Cre^{+/-}/*Mertk*^{fl/wt} mice had a median grade of 2.5 compared to a median grade of 1.0 in female CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice ($p=0.0006$). No difference in disease onset or clinical severity index was observed between male and female CD11c-Cre^{-/-}/*Mertk*^{fl/fl} control mice (**Fig. 4.1h**).



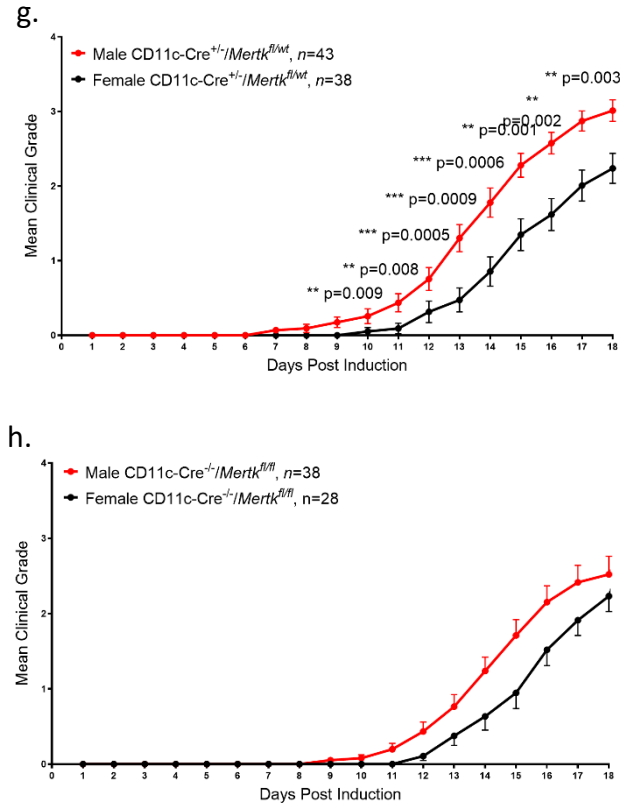


Figure 4.1. *Mertk* conditional deletion exacerbates clinical outcomes in EAE. **a.** Higher mean clinical grade was observed in CD11c-Cre^{+/+}/Mertk^{fl/wt} mice compared to CD11c-Cre^{+/+}/Mertk^{fl/fl} controls at 15 dpi, (Mann-Whitney U test, **p*=0.016); **b.** No difference was observed in female CD11c-Cre^{+/+}/Mertk^{fl/wt} mice compared to female CD11c-Cre^{+/+}/Mertk^{fl/fl} controls at any time point; **c.** All observed differences in clinical outcome were attributable to male CD11c-Cre^{+/+}/Mertk^{fl/wt} mice compared to male CD11c-Cre^{+/+}/Mertk^{fl/fl} controls. Enhanced disease was observed at days 12 through 14 dpi (Mann-Whitney U test, ****p*<0.014 for all time points); **d-f.** Survival analysis confirmed earlier incidence of EAE in CD11c-Cre^{+/+}/Mertk^{fl/wt} mice compared to CD11c-Cre^{+/+}/Mertk^{fl/fl} controls (Hazard ratio 0.62, 95% CI 0.40 – 0.96, Log-rank (Mantel-Cox) test **p*=0.03). As with clinical severity, this result was entirely attributable to male animals (Hazard ratio 0.52, 95%CI 0.25 – 0.93, Log-rank (Mantel-Cox) test **p*=0.03). No difference in timing of disease incidence was observed in female mice (Hazard ratio 1.0, 95% CI 0.55 – 1.91); **g.** Enhanced disease was observed in male CD11c-Cre^{+/+}/Mertk^{fl/wt} compared to female CD11c-Cre^{+/+}/Mertk^{fl/wt} mice at days 10 through 18 dpi (Mann-Whitney U test, ****p*<0.008 for all time points); **h.** No difference in clinical severity index was noted in male CD11c-Cre^{+/+}/Mertk^{fl/fl} mice compared to female CD11c-Cre^{+/+}/Mertk^{fl/fl} littermates.

4.2.1 Sex-Specific Differences in EAE Outcome Moderate Over Time.

The striking difference in clinical outcomes observed in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice diminished over time with subsequent cohorts. Specifically, in the first three cohorts exposed to EAE, male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice experienced more severe clinical disease compared to CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls at 12 through 15 dpi. At 15 dpi male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice had a median grade of 2.5 compared to a median grade of 1.0 in male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls ($p=0.04$) (**Fig. 4.2a**). In addition, in the first three cohorts exposed to EAE survival analysis confirmed earlier incidence of symptoms in male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice compared to male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls (Hazard ratio 0.34, 95% CI 0.15 – 0.80, Log-rank (Mantel-Cox) test $p=0.01$) (**Fig. 4.2c**). In the subsequent five cohorts, no differences in the severity of EAE nor in the timing of the onset of EAE symptoms were observed (**Fig. 4.2b,d**).

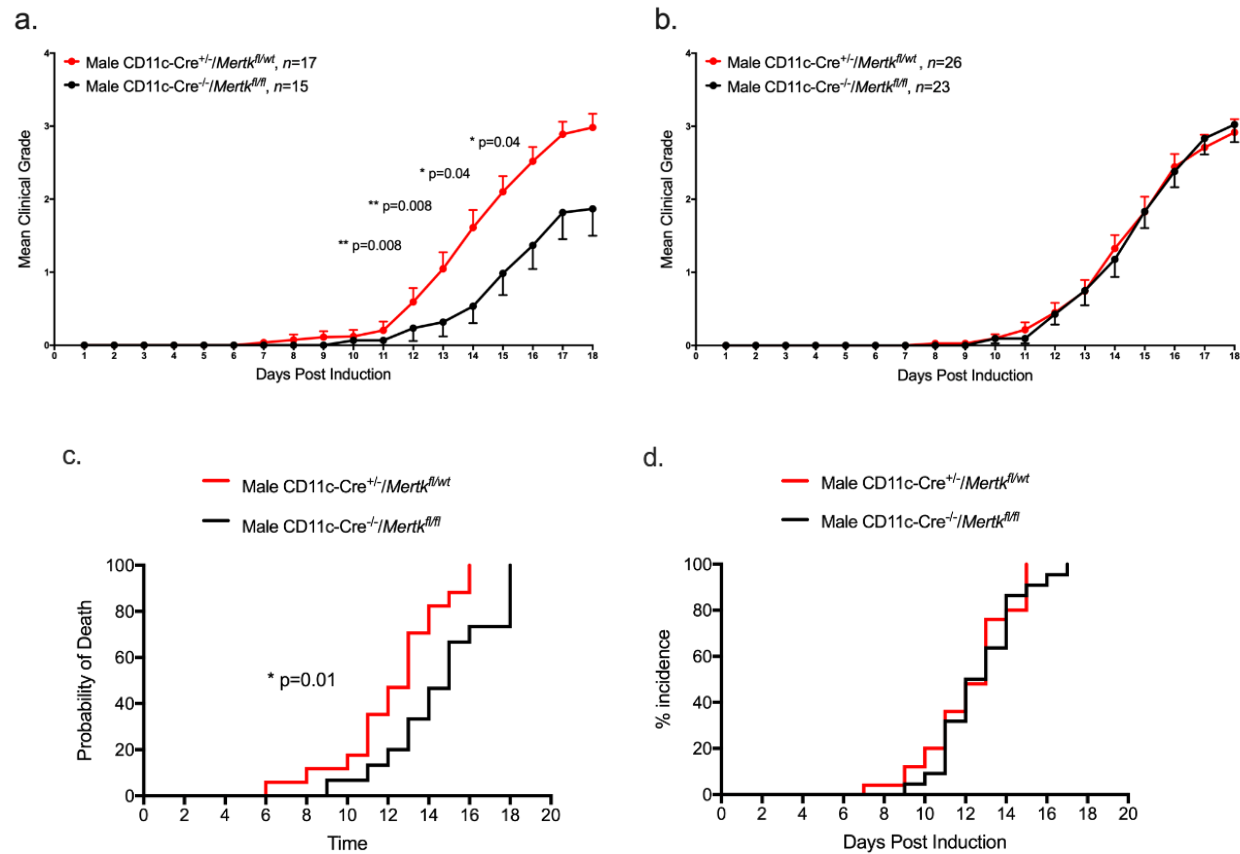


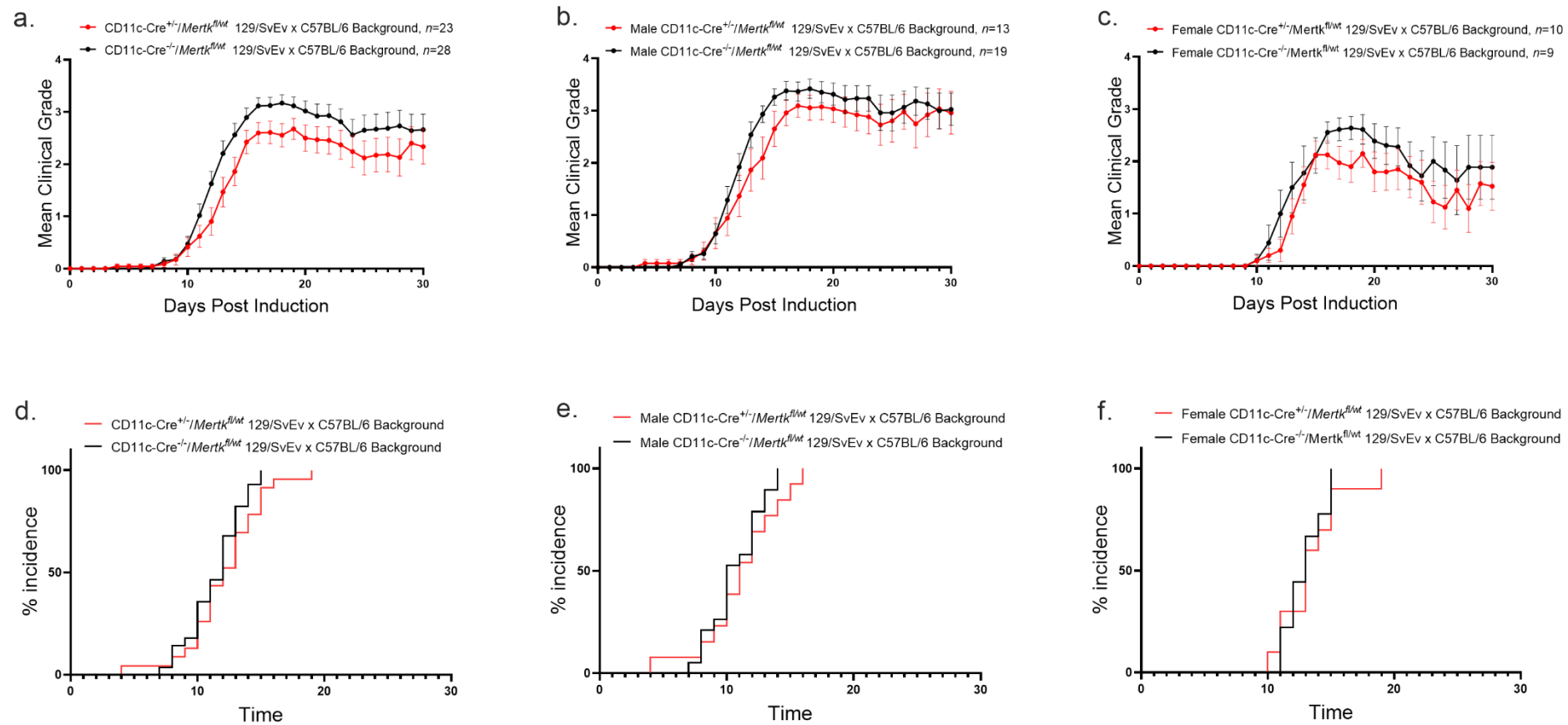
Figure 4.2. Loss of clinical phenotype in CD11c-Cre^{+/-}/Mertk^{fl/wt} animals over time. **a.** More severe clinical grades observed in male CD11c-Cre^{+/-}/Mertk^{fl/wt} mice compared to male CD11c-Cre^{-/-}/Mertk^{fl/fl} controls in first three cohorts exposed to EAE at 13-16 dpi, (Mann-Whitney U test, *⁻**p<0.04 for all time points); **b.** In subsequent five cohorts, no difference in clinical grades observed in male CD11c-Cre^{+/-}/Mertk^{fl/wt} mice compared to male CD11c-Cre^{-/-}/Mertk^{fl/fl} controls exposed to EAE from 2017** onwards; **c.** Survival analysis confirmed earlier incidence of EAE in CD11c-Cre^{+/-}/Mertk^{fl/wt} mice compared to CD11c-Cre^{-/-}/Mertk^{fl/fl} controls (Hazard ratio 0.34, 95% CI 0.15 – 0.80, Log-rank (Mantel-Cox) test *p=0.01) in first three cohorts exposed to EAE; **d.** In subsequent five cohorts, no difference in timing of onset of EAE symptoms was observed.

4.2.2 Backcrossing With 129/SvEv Mice Does Not Restore a More Severe EAE Clinical Phenotype in CD11c-Cre^{+/-}/Mertk^{fl/wt} Mice.

The development of the CD11c *Mertk* conditional knock-out was performed in mice from mixed genetic backgrounds. Specifically, the insertion of flanking loxP sites at the location of the *Mertk* gene was undertaken in 129/SvEv strains. Conversely, the construction of the Cre driver line was performed in C57BL/6 mice. Animals from this mixed background were subject to accelerated backcrossing to a uniform C57BL/6 background before undertaking the later EAE cohorts. I hypothesised that the loss of clinical phenotype seen in *Mertk* heterozygous deleted mice reflected the abrogation of a disease modifier that was initially present in animals from a mixed 129/SvEv and C57BL/6 lineage that was not present after complete backcrossing to the C57BL/6 genotype. Accordingly, existing CD11c-Cre^{+/-}/Mertk^{fl/fl} mice were backcrossed with wild-type 129/SvEv animals in a bid to reintroduce an unidentified disease modifier present in those animals.

This breeding yielded CD11c-Cre^{+/-}/Mertk^{fl/wt} and CD11c-Cre^{-/-}/Mertk^{fl/wt} animals at Mendelian frequencies. Animals from the first generation of this breeding were exposed to EAE as set out above. Unlike earlier cohorts which were terminated at 18 dpi in order to perform immunohistochemical analysis at disease nadir, these cohorts were followed for 30 days.

No difference in clinical severity was observed (**Fig. 4.3a**). Survival analysis did not identify a difference in the timing of disease incidence among mixed background CD11c-Cre^{+/-}/Mertk^{fl/wt} and CD11c-Cre^{-/-}/Mertk^{fl/wt} animals (**Fig. 4.3d**). Subgroup analysis stratified by sex did not reveal sex-specific differences in disease severity or timing of symptom onset (**Fig. 4.3b,c,e,f**). Furthermore, immunohistochemical analysis of inflammatory infiltrates in the lumbar spinal cord of EAE-treated mice did not identify a sex- or genotype specific differences. Specifically, no sex-based difference in cells counts of either CD3^{+ve} or CD11c^{+ve} cells were identified within mixed 129/SvEv and C57BL/6 genotypes (**Fig. 4.3o,p**).



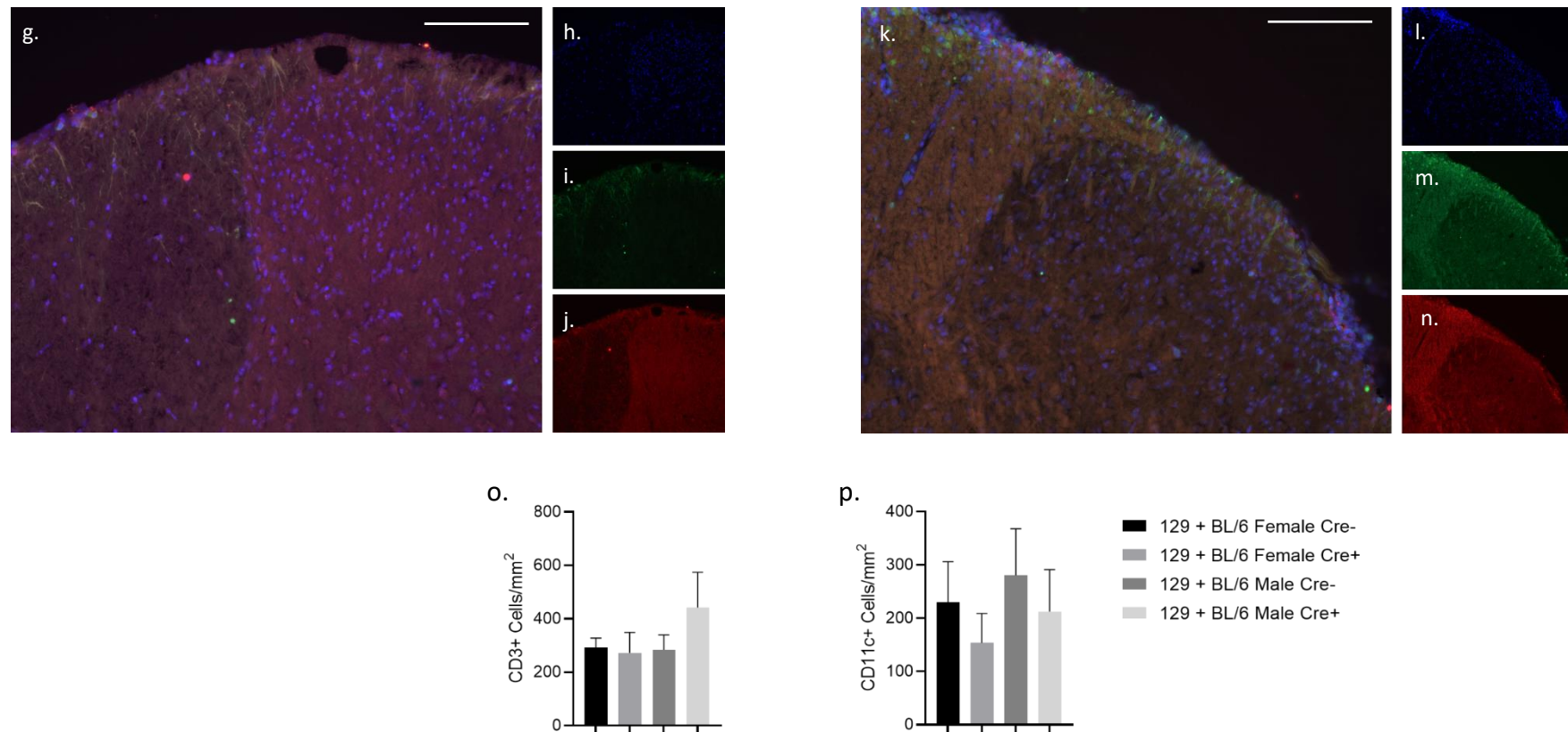


Figure 4.3. Backcrossing with 129/SvEv mice does not restore more severe EAE clinical phenotype. **a-c.** No difference in clinical grades was observed in CD11c-Cre^{+/-}/Mertk^{fl/wt} mice compared to CD11c-Cre^{-/-}/Mertk^{fl/fl} controls from mixed 129/SvEv and C57BL/6 backgrounds; **d-f.** No difference in the timing of disease onset was observed in sex combined cohorts (Hazard ratio 1.6, 95% CI 0.85 – 3.16), male only cohorts (Hazard ratio 1.55, 95% CI 0.68 – 3.58) or female only cohorts (Hazard ratio 1.40, 95% CI 0.45 – 4.37); **g-j.** Representative photomicrograph of mouse dorsal column in lumbar spine from 129/SvEv and C57BL/6 mixed background CD11c-Cre^{-/-}/Mertk^{fl/wt} mouse stained for DAPI (h), CD3 (i) and CD11c (j); **k-n.** Representative micrograph of mouse dorsal column in lumbar spine from 129/SvEv and C57BL/6 mixed background CD11c-Cre^{+/-}/Mertk^{fl/wt} mouse stained for DAPI (l), CD3 (m) and CD11c (n); **o-p.** Cell counts per mm² of CD3⁺ and CD11c⁺ cells in the lumbar spinal cord of mixed background 129/SvEv and C57BL/6 CD11c-Cre^{+/-}/Mertk^{fl/wt} and CD11c-Cre^{-/-}/Mertk^{fl/wt} animals stratified by sex; analysis performed at 30 dpi. Scale bars = 100 μm

4.2.3 Homozygous Deletion of *Mertk* Does Not Result in Exacerbation of EAE in C57BL/6 Mice

I hypothesised that *Mertk* homozygous deletion would result in a more severe clinical manifestation of EAE compared to both Cre- control and heterozygote deleted mice, as enhanced disease severity with relative dose reduction of MERTK was anticipated. Accordingly, a subsequent analysis was undertaken in CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice to examine whether the previously observed phenotype in male mice could be re-established. As noted, CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice did not breed at expected Mendelian frequencies and all animals used for experimentation were progeny of a single CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mother.

The clinical phenotype evident in the first three cohorts of *Mertk* heterozygote deleted animals was not replicated in this group. Although more severe clinical disease was evident in male and female CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice at 18 dpi ($p=0.004$), earlier disease onset and more severe clinical grades throughout the experimental period were not identified (**Fig. 4.4a**). Stratification of clinical severity index did not identify sex-specific differences. (**Fig. 4.4b, c**).

Similarly, survival analysis did not identify a significant difference in the timing of disease onset in CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice compared to CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls (Hazard ratio 0.81, 95% CI 0.49 – 1.34) (**Fig. 4.4d**). No difference in timing of disease onset was observed in male mice (Hazard ratio 0.57, 95% CI 0.27 – 1.20) or female mice (Hazard ratio 0.94, 95% CI 0.45 – 1.97) (**Fig. 4.4e,f**).

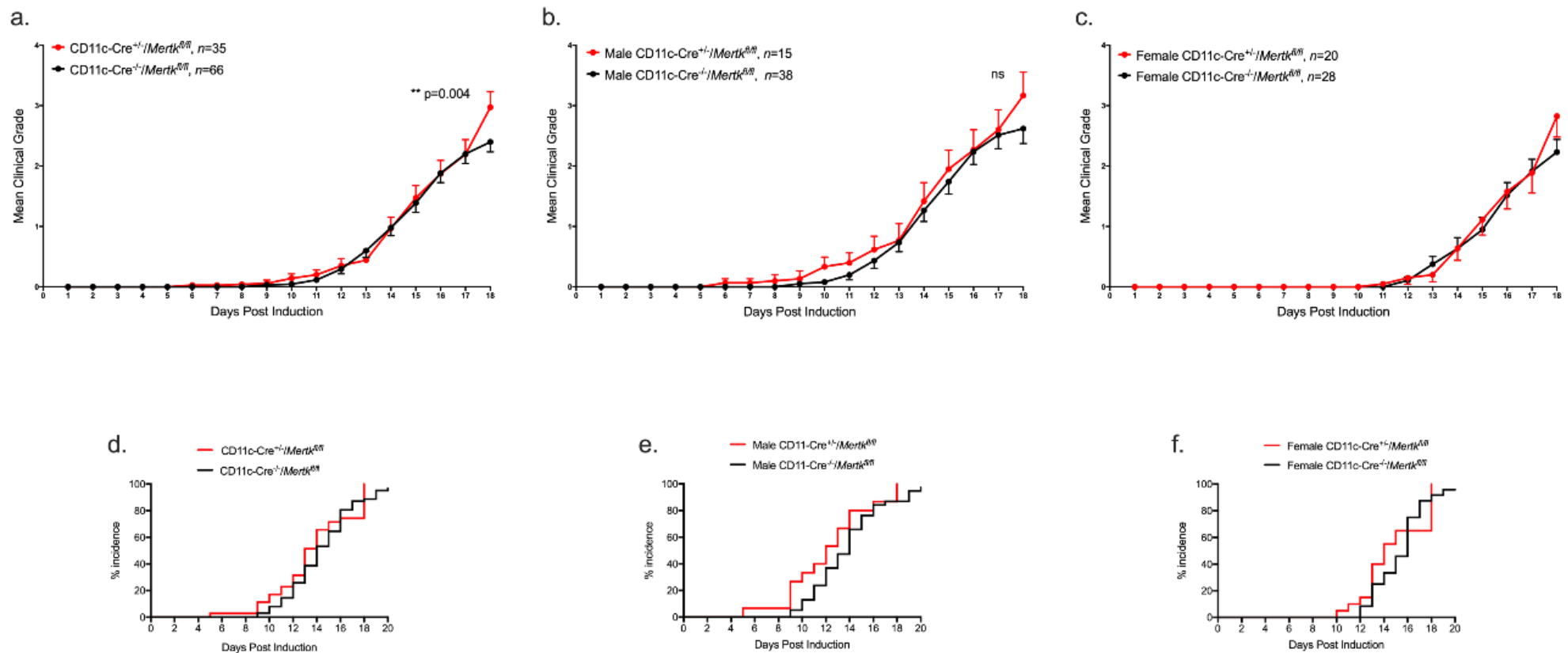


Figure 4.4. More severe clinical phenotype not observed in CD11c-Cre^{+/-}/Mertk^{fl/fl} animals. **a.** Higher mean clinical grade was observed in CD11c-Cre^{+/-}/Mertk^{fl/fl} mice compared to CD11c-Cre^{-/-}/Mertk^{fl/fl} controls only at 18 dpi (Mann-Whitney U test, **p=0.004); **b, c.** No difference in EAE severity was observed in male or female CD11c-Cre^{+/-}/Mertk^{fl/fl} mice compared to male or female CD11c-Cre^{-/-}/Mertk^{fl/fl} controls at any time point; **d-f.** Survival analysis did not identify earlier incidence of EAE in CD11c-Cre^{+/-}/Mertk^{fl/fl} mice compared to CD11c-Cre^{-/-}/Mertk^{fl/fl} controls (Hazard ratio 0.81, 95% CI 0.49 – 1.34). No difference in timing of disease onset was observed in male mice (Hazard ratio 0.57, 95% CI 0.27 – 1.20) or female mice (Hazard ratio 0.94, 95% CI 0.45 – 1.97).

4.2.4 Microsatellite and Quantitative PCR Analyses Identified a Common *Tyro3* Allele Among CD11c-Cre^{+/-}/*Mertk*^{fl/wt} Animals

Tyro3 and *Mertk* lie in close proximity on mouse Chromosome 2, within 10 Mb. There is evidence that the *Tyro3* gene in 129/SvEv animals is expressed at lower levels than in C57BL/6 mice. This has been reported to have clinical and histological consequences in other disease models where *Mertk* is abrogated in 129/SvEv and C57BL/6 mice, leading to marked differences in disease outcome for these two strains.¹⁶⁰

I was hypothesised that at some stage during repeated breeding cycles the low expressing *Tyro3* allele from the 129/SvEv background was replaced with the higher expressing C57BL/6 allele, thereby compensating for the conditional deletion of *Mertk* in later cohorts where a more severe clinical phenotype in males was not observed. This hypothesis would explain both the loss of phenotype over time in male CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice and the absence of more severe clinical disease in CD11c-Cre^{+/-}/*Mertk*^{fl/fl} as those animals were only available for experimentation after the completion of all heterozygous deleted cohorts.

To test this hypothesis, microsatellite analysis of the relevant region of mouse Chromosome 2 was undertaken using DNA from CD11c-Cre^{+/-}/*Mertk*^{fl/fl} animals that were littermates of male animals that experienced the more severe clinical phenotype of EAE. Samples were also analysed from animals in later cohorts where the more severe male phenotype was not observed.

Notably, the expected allele size at D2Mit420 (**Fig. 4.5**), which lies proximal to both *Tyro3* and *Mertk* was common among both phenotypically affected and unaffected cohorts, indicating that the region proximal to *Tyro3* and *Mertk* has not undergone recombination and retains 129/SvEv alleles. This suggests that the modifier of clinical outcomes responsible for the observed difference in the severity of EAE over time is not the low expressing *Tyro3* allele from 129/SvEv lineage.

<i>Sample Name</i>	<i>Marker</i>	<i>Allele 1</i>	<i>Allele 2</i>	<i>Size 1</i>	<i>Size 2</i>			
Affected Cohort Male	D2Mit229	229	229	228.8	228.8			
Affected Cohort Female	D2Mit229	229	229	228.7	228.7			
Unaffected Cohort Male	D2Mit229	229	229	228.66	228.66			
Unaffected Cohort Female	D2Mit229	229	229	228.7	228.7			
						<i>Expected Allele Sizes</i>		
						<i>Marker</i>	<i>C57BL/6</i>	<i>129/SvEv</i>
Affected Cohort Male	D2Mit265	Failed	Failed	Failed	Failed	D2Mit229	229	237
Affected Cohort Female	D2Mit265	Failed	Failed	Failed	Failed	D2Mit265	107	111
Unaffected Cohort Male	D2Mit265	Failed	Failed	Failed	Failed	D2Mit420	125	88
Unaffected Cohort Female	D2Mit265	Failed	Failed	Failed	Failed	D2Mit48	194	184
Affected Cohort Male	D2Mit420	88	88	87.58	87.58	D2Mit83	114	
Affected Cohort Female	D2Mit420	88	88	87.49	87.49	D2Mit91	194	184
Unaffected Cohort Male	D2Mit420	88	88	87.5	87.5			
Unaffected Cohort Female	D2Mit420	88	88	87.44	87.44			
Affected Cohort Male	D2Mit48	194	194	194.13	194.13			
Affected Cohort Female	D2Mit48	194	194	193.95	193.95			
Unaffected Cohort Male	D2Mit48	194	194	194	194			
Unaffected Cohort Female	D2Mit48	194	194	193.89	193.89			
Affected Cohort Male	D2Mit83	114	114	114.23	114.23			
Affected Cohort Female	D2Mit83	114	114	114.12	114.12			
Unaffected Cohort Male	D2Mit83	114	114	114.22	114.22			
Unaffected Cohort Female	D2Mit83	114	114	114.05	114.05			
Affected Cohort Male	D2Mit91	194	194	194.07	194.07			
Affected Cohort Female	D2Mit91	194	194	193.98	193.98			
Unaffected Cohort Male	D2Mit91	194	194	194.08	194.08			
Unaffected Cohort Female	D2Mit91	194	194	193.86	193.86			

Figure 4.5. Microsatellite analysis of regions flanking *Tyro3* in CD11c-Cre^{+/-}/*Mertk*^{fl/fl} animals from phenotypically affected and unaffected cohorts. The expected allele size at D2Mit420 indicates common 129/SvEv ancestry among both affected and unaffected cohorts, suggesting that the low expressing 129/SvEv *Tyro3* allele is conserved among both groups.

To corroborate the outcome of the microsatellite analysis, the relative expression of *Tyro3* was assessed at the RNA level by qPCR analysis. Brain and spleen homogenates were used for RNA isolation. Tissue was derived from wild-type 129/SvEv, CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice from a mixed 129/SvEv background, CD11c-Cre^{+/-}/*Mertk*^{fl/fl} from phenotypically affected cohorts, CD11c-Cre^{-/-}/*Mertk*^{fl/fl} from phenotypically unaffected cohorts and wild-type C57BL/6 mice.

As expected, 129/SvEv mice demonstrated lower relative expression of *Tyro3* in brain homogenates compared to C57BL/6 animals (**Fig. 4.6a**). In addition, all *Mertk* floxed mice demonstrated lower relative expression of *Tyro3*, confirming the microsatellite

analysis and indicating that the low expressing *Tyro3* allele from the original 129/SvEv strain used to create the *Mertk* floxed mouse is well conserved (**Fig. 4.6b**). In keeping with prior analyses,¹⁶¹ negligible splenic expression of *Tyro3* was identified.

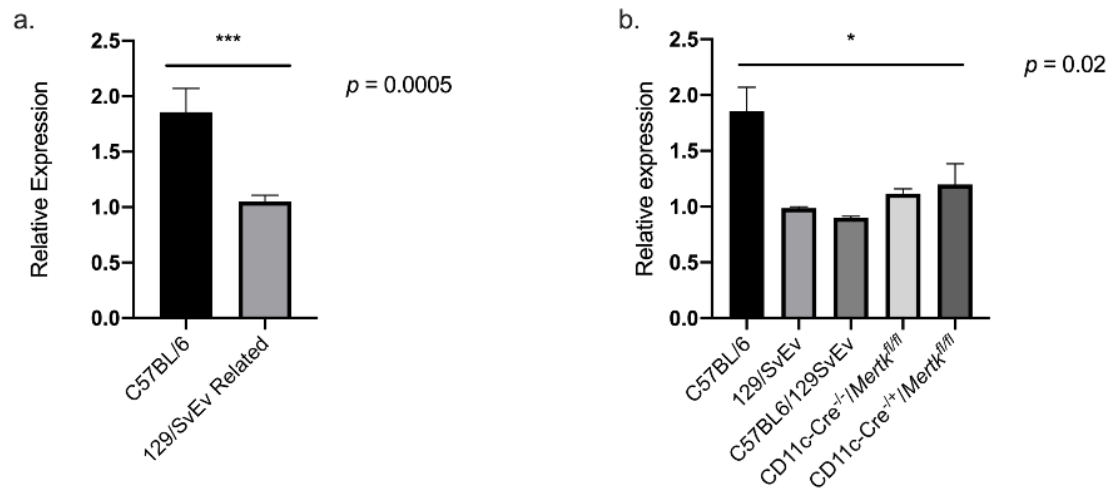


Figure 4.6. Relative expression of *Tyro3* in mouse brain at the RNA level. **a.** The relative expression of *Tyro3* in C57BL/6 mouse brain was significantly greater when compared to combined 129/SvEv related strains by qPCR analysis (Student's T-test, *** $p=0.0005$); **b.** Consistently significant reductions in *Tyro3* expression in all 129/SvEv related subgroups compared to C57BL/6 mice (One-way ANOVA, * $p=0.02$). Negligible levels of *Tyro3* were identified in the spleen (not shown).

4.3 *Mertk* Deficiency in CD11c⁺ Microglia Does Not Induce Microgliosis During Cuprizone-Induced Demyelination.

It was previously reported that a subset of microglia within the demyelinated brain express CD11c¹⁶² and that global deficiency of the TAM ligand GAS6 leads to enhanced numbers of microglia in a cuprizone model of demyelination.¹⁰⁵ It was therefore important to determine whether *Mertk* deficiency in CD11c⁺ microglia might also induce the activation of these cells, potentially altering either clinical or immunohistochemical outcomes of cuprizone exposure in *Mertk* cKO cohorts. If changes in *Mertk* deficient cohorts were seen following cuprizone administration, this would suggest that a CNS-derived immune phenomenon might account for the sexually dimorphic exacerbation of EAE noted in male heterozygous deleted animals.

Mice were fed mouse chow containing 0.2% (w/v) cuprizone for three weeks prior to analysis. Although many cuprizone protocols provide for a five-week administration

period, a shortened experimental paradigm was selected in order to perform immunohistochemical analysis during the period of most substantial microgliosis, which typically occurs during week three of cuprizone administration.

Immunohistochemical analyses of the rostral corpus callosum of CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice and CD11c-Cre^{-/-}/*Mertk*^{fl/fl} controls were performed by staining for the marker ionised calcium binding adapter molecule 1 (IBA1) in concert with surface antigen MAC3 (MAC3) to identify activated phagocytic microglia.¹⁶³

No obvious phenotypic differences were noted between genotypes or sexes during the experiment. No genotype or sex-specific difference in the number of activated microglia in male and female CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice was identified by immunohistochemical analysis) (**Fig. 4.7i**).

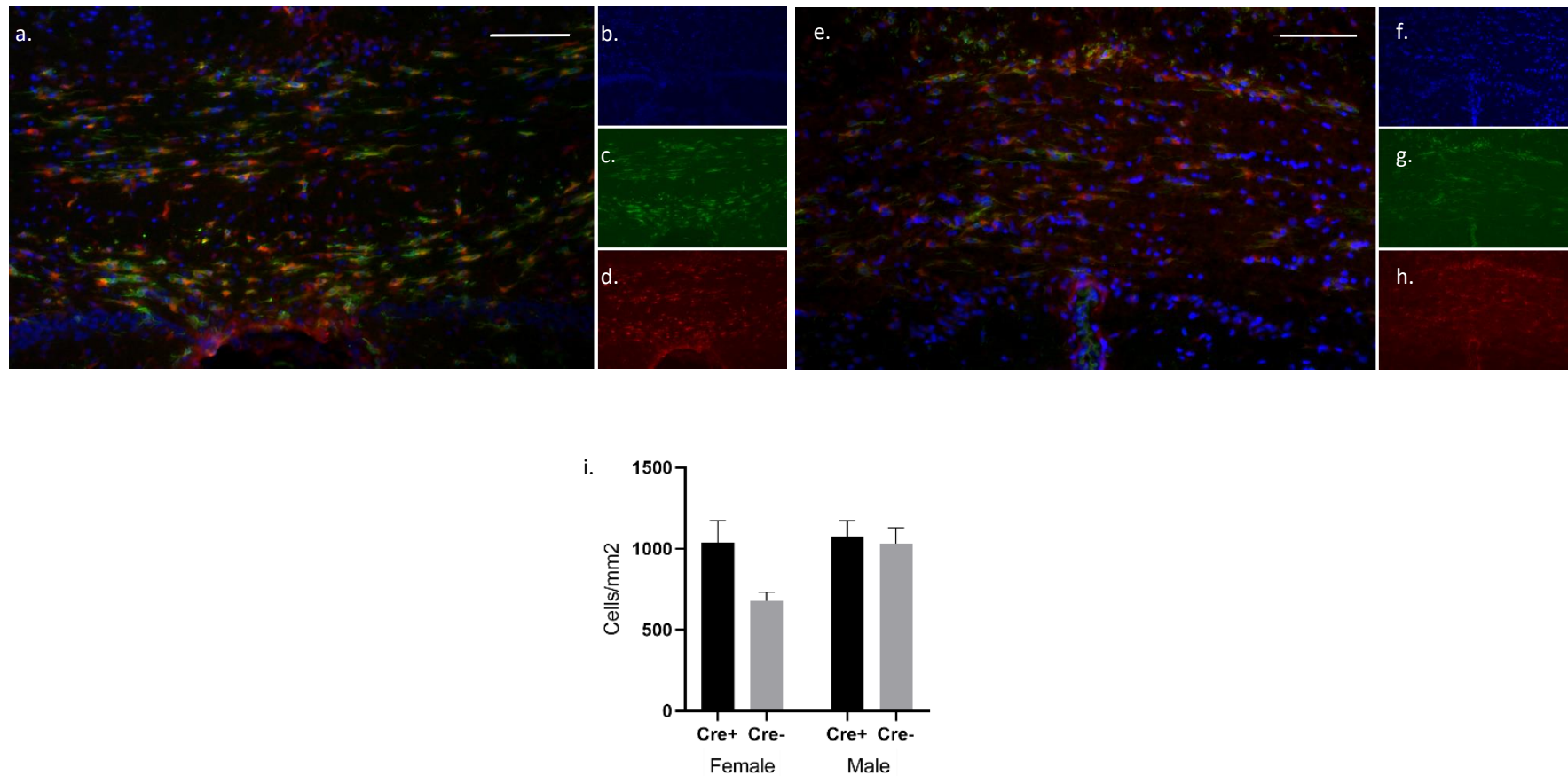


Figure 4.7. MERTK deficiency in CD11c⁺ microglia does not induce microgliosis during cuprizone-induced demyelination. **a-d.** Representative photomicrograph of rostral mouse corpus callosum in CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mouse stained for DAPI (b), IBA1 (c) and MAC3 (d); **e-h.** Representative photomicrograph of rostral mouse corpus callosum in CD11c-Cre^{-/-}/*Mertk*^{fl/wt} mouse stained for DAPI (f), IBA1 (g) and MAC3 (h); **i.** No sex-specific difference observed in the number of activated microglia between male (1038 ± 470 cells/mm²) and female (1074 ± 570 cells/mm²) CD11c-Cre^{+/+}/*Mertk*^{fl/wt} mice. Scale bars = 50 μm.

4.4 Discussion

This chapter sets out an analysis of central and peripherally mediated models of demyelination in a novel conditional knock-out mouse targeting the expression of *Mertk* in CD11c⁺ cells. This platform provides a powerful new tool for interrogating the role of *Mertk* in modulating immune responses by dendritic cells. Animals both hetero- and homozygous for *Mertk* deletion are available under this paradigm, potentially allowing for the assessment of dose-related effects of *Mertk* expression. As noted in Chapter 3, flow cytometric analysis of immune cell subtypes demonstrates that conditional deletion of *Mertk* does not alter immune populations under physiological conditions, suggesting that deletion of *Mertk* from CD11c⁺ cells does not impair immune system development. In addition, *Mertk* deletion from CD11c⁺ cells does not result in obvious phenotypic or behavioural differences compared to wild-type mice.

The abrogation of *Mertk* expression resulted in exacerbated clinical outcomes of EAE in male mice in early cohorts. While *Mertk*'s role in the modulation of immune responses is well established and its association with the development of central inflammatory demyelination in humans is now accepted, the data in this chapter confirm a role for *Mertk* in the development of the most frequently employed mouse model of multiple sclerosis. The identification of sexually dimorphic responses to EAE induction has potentially important parallels with experiences in human disease; male MS patients more rapidly accumulate neurological deficits and are more likely to experience disease progression after diagnosis with multiple sclerosis.¹⁵⁹

The observation of phenotypic difference in EAE outcome was surprising in light of the absence of sex-specific differences in either dendritic cell expression of *Mertk* or immunohistochemical analyses of spinal cord inflammatory infiltrates in animals euthanised at disease peak. Given the inherent variability of EAE as an experimental paradigm,^{164, 165} this result was confirmed by inducing EAE in eight separate cohorts with grading performed by multiple researchers blinded to animal genotype.

What mechanism might account for worsened EAE outcomes in *Mertk* deficient animals? A role for *Axl* in the suppression of T cell responses is well established,¹³⁹ though less is known about *Mertk*'s capacity for T cell modulation. It is known that ProS-mediated T cell regulation of dendritic cell activation is attenuated when T cells are co-cultured with dendritic cells deficient in *Axl* and *Mertk* function.¹⁵⁵ As ProS is a ligand for *Mertk* but not *Axl* it would appear that dendritic cell *Mertk* plays a key role in sculpting T cell responses and that this might be particularly evident in the activation stage of EAE when antigen is first presented to circulating T cells. In addition to their immunomodulatory function, dendritic cells are capable of highly efficient antigen presentation. Under physiological conditions, *Mertk* plays an important role in efferocytosis and the removal of cellular debris. *Mertk* signalling in this context results in the downregulation of pro-inflammatory cytokines and the suppression of antigen presentation via MHC-II.¹⁵⁵ The loss of *Mertk* in dendritic cells may result in altered antigen presentation and upregulation of proinflammatory cytokines, leading to enhanced T cell engagement in the demyelinating pathology of EAE and worsened clinical outcomes for affected animals. Although T cell stimulation might result in increased ProS production, without the ability to signal through dendritic cell-bound *Mertk*, anti-inflammatory influences cannot be propagated, leading to a cycle of maladaptive immune activation without the presence of balancing regulatory inputs.

This perspective is supported by preliminary data generated by others in my laboratory group. Specifically, using cells from *CD11c-Cre^{+/-}/Mertk^{fl/fl}* mice, colleagues have identified that *Mertk* deficiency in *CD11c^{+ve}* results in increased T cell proliferation following OVA-II peptide administration (unpublished data communicated with the permission of J. Field). This suggests that *Mertk* signals through dendritic cells in their role as professional antigen presenting cells to downregulate T cell mediated responses and that this effect is impaired in settings of reduced *Mertk* expression. Further interrogation of this putative mechanism is required in light of the concomitant finding that *Mertk* deletion did not result in changes in MHC-II expression which might have been expected, *a priori*.

While EAE exacerbation in MERTK deficient male mice was noted in early cohorts, this phenotype was lost over time, despite various experimental measures to restore this finding. The loss of phenotype does not appear to be related to inter-observer variability. A single trainer was used to instruct all experimental staff involved in the induction, handling and EAE grading of all cohorts. In addition, in order to ensure consistency of EAE scoring, a blinded EAE grader was engaged to “spot-check” EAE scores on a number of occasions throughout the course of these experiments. A very high degree of consistency in EAE assessment was identified.

Myelin oligodendrocyte glycoprotein must be combined with Complete Freund’s Adjuvant prior to each EAE induction and as the MOG₃₅₋₅₅ peptide is synthesised, variations in its immunogenicity can lead to inconsistency in EAE outcomes.¹⁶⁶ In order to control for this possibility, MOG₃₅₋₅₅ peptide was purchased from a single supplier. To reduce the possibility of a large volume of low potency MOG₃₅₋₅₅ peptide altering outcomes with ageing of the reagent, only small quantities were provided just prior to experimental induction. In addition, MOG₃₅₋₅₅ peptide was reconstituted from lyophilised powder and combined with complete Freund’s adjuvant only on the day of induction. Only a volume sufficient to induce the number of animals for experimentation was prepared and any excess was discarded. Accordingly, experimental factors are likely not the source of the change in phenotype observed over time.

Cohorts of inbred experimental mice are subject to genetic drift and as a result spontaneous mutations in apparently unrelated genes can cause unexpected loss or gain of phenotypic qualities over time.¹⁶⁷ This can be overcome by embryo freezing at the start of experimental paradigm so that a genetic profile can be “restored” if drift is suspected but this resource was not available in the context of the current experiments.

A change in the gut microbiome may also result in changes in phenotypic outcome. Gut microbiota can alter the barrier function of the intestinal tract and also modulate responses to ingested drugs and therapeutics. Of more relevance to the current

experiments, gut microbiota also appear to influence distant immune responses in paradigms where intestinal diseases are not under interrogation.¹⁶⁸ Accordingly, a change in the commensal gut microenvironment might also account for the loss of phenotype observed in these cohorts.

Finally, it should be noted attempts to recapitulate the genomics of the original animal cohorts through the reintroduction of wild-type 129/SvEv mice do not directly mirror the genetic conditions of those first experiments. Specifically, in the original animals the floxed allele was contributed from the 129/SvEv strain while the Cre-driver was provided by C57BL/6 mice. In the reconstituted animals bred in a bid to restore the experimental phenotype, both the floxed allele and the Cre-driver were associated with the C57BL/6 animals, with the former reflecting the genetic recombination on chromosome 2 that occurred with backcrossing onto the C57BL/6 background. This raises the possibility of an unidentified modifier present in 129/SvEv mice that is not found in C57BL/6 animals – or vice versa. Experiments using a congenic approach where the floxed allele is directly introduced into Cre-driver expressing animals may be required to interrogate this possibility further.

Cuprizone intoxication induces highly reproducible episodes of central demyelination in discrete brain regions, including the corpus callosum. Histologically, these lesions resemble Pattern III and IV lesions, as described by Lucchinetti and colleagues.^{68, 169} They are notable for oligodendrocyte loss and subsequent demyelination without adaptive immune infiltrate, potentially due to cuprizone's toxic effect on T cells.^{170, 171} As microglia may also express CD11c and TAM receptors are required for microglial clearance of apoptotic cells,¹⁷² it was important to examine if conditional deletion of MERTK in CD11⁺ cells resulted in phenotypic or immunohistochemical changes following cuprizone intoxication.

No phenotypic difference was noted between CD11c-Cre^{+/+}/*Mertk*^{fl/wt} or CD11c-Cre^{-/-}/*Mertk*^{fl/fl} mice following three weeks of cuprizone supplementation. In addition,

staining specific for activated microglia did not identify a difference in cellular responses to toxic demyelination. Accordingly, I hypothesise that MERTK expressed by CD11c⁺ve microglia is not central to the outcome of cuprizone-mediated demyelination. Furthermore, studies examining the role of microglia in the removal of apoptotic debris have focused on different microglial subsets.¹⁷² The absence of phenotypic or immunohistochemical differences following microglial deletion of *Mertk* in CD11c⁺ve cells and following cuprizone intoxication supports a focus on peripheral immune activation via CD11c⁺ve dendritic cells in response to EAE.

However, a hypothesis that proposes a key role for dendritic cell expressed *Mertk* in the modulation of models of inflammatory demyelination is challenged by substantial caveats. As already noted, CD11c is also expressed by microglia and these cells are activated in the brains and spinal cords of mice during EAE. Could EAE exacerbation in *Mertk* deficient animals reflect impaired microglial phagocytosis, with an increasing burden of autoantigen in the CNS leading to a cycle of escalating immune activation? The finding that cuprizone-challenged CD11c-Cre^{+/+}/*Mertk*^{fl/fl} males do not show increased levels of microglial activation compared to CD11c-Cre^{-/-}/*Mertk*^{fl/fl} males is in keeping with prior observations¹⁷³ and suggests against such a conclusion. However, microglial responses to cuprizone intoxication may not mirror those in EAE as the mechanisms of demyelination are very different in the two models. In particular, the relative absence of peripheral immune infiltrates following cuprizone administration may create a “sterile” immune environment in which there is relatively limited exposure to inflammatory cytokines and other mediators of enhanced immune responses. In that setting *Mertk* abrogation in microglia may not have clinical or histochemical consequences that would, however, be unmasked in a model that involves peripheral immune activation. As an important caveat to this theory, it is acknowledged that cuprizone intoxication does induce some degree of monocyte-derived macrophage infiltration and these cells also frequently express CD11c. Further investigation is clearly needed to determine if dendritic cells or microglia are the relevant effector cells responsible for the phenotype seen in early cohorts following EAE induction. An

intriguing potential model involves the use of dendritic cell transfer experiments to assess the anti-inflammatory capacity of *Mertk* deficient dendritic cells to ameliorate the clinical effects of EAE. In WT mice, repeated immunisation with MOG antigen produces a population of tolerogenic CD11b⁺CD11c⁺ dendritic cells that can dramatically improve outcomes following transfer to animals at EAE disease peak.⁸⁶ Loss of that ability in *Mertk* deficient dendritic cells would confirm a role for that cell population in the nascent clinical phenotype identified in this chapter.

The apparent sexual dimorphism in EAE severity in *Mertk* deficient animals requires further investigation. As set out in chapter 3, sex-specific differences in the efficiency of *Mertk* deletion were not identified at the RNA or protein level. *Mertk* deletion did not alter resting immune cell populations in a sex-specific manner. Sex did not change levels of *Axl*, *Tyro3* or *Mertk* expression in the lumbar spinal cord following EAE induction, with the exception of *Mertk* in a subset of male mice experiencing relatively severe EAE symptoms. No sex-specific differences were seen in CD3⁺ T cell and CD11c⁺ dendritic cell infiltration of the lumbar spinal cord of mixed background 129/SvEv x C57BL/6 mice following EAE induction. In short, to date a quantifiable molecular or cellular variable to account for the startlingly more severe clinical phenotype observed in early cohorts of CD11c-Cre^{+/+}/*Mertk*^{fl/wt} male mice has not been identified.

Oestrogen and other female sex hormones are known to exert anti-inflammatory effects and multiple groups have reported their beneficial effects on the clinical outcomes of EAE.^{19-21, 174-176} In women with MS, relapse rates are greatly reduced during pregnancy, when serum oestrogen levels are elevated and a bias towards to Th2 mediated immunity is observed.^{16, 177, 178} Exposure to tamoxifen, a small-molecule estrogen receptor modulator, inhibits the ability of dendritic cells to stimulate CD4⁺ T cells by downregulating MHC-II expression, resulting in T cell anergy following peptide stimulation.²⁰ Oestrogen may provide a compensatory anti-inflammatory mechanism that is able to abrogate the effects of *Mertk* deficiency in female mice that is not present in male animals. A physiological bias towards Th2 responses in female mice may

overcome the loss of ProS-mediated T cell negative feedback on dendritic cell activation. To interrogate this hypothesis, repeat experiments in oophorectomised CD11c-Cre^{+/-}/*Mertk*^{fl/wt} and CD11c-Cre^{+/-}/*Mertk*^{fl/fl} mice compared to female mice treated with sham surgery could be undertaken. I hypothesise that oophorectomised mice will experience a disease course that closely matches the experience of male *Mertk* deficient mice. Of course, this experimental program hinges first on the re-establishment of the more severe clinical phenotype in CD11c-Cre^{+/-}/*Mertk*^{fl/wt} mice.

The analysis presented in Chapter 3 demonstrates that resting levels of TAM receptor and ligand expression in the lumbar spinal cord are equal among male and female C57BL/6 mice. However, in the periphery, *Axl* expression by female monocytes is higher in the unchallenged state but not during EAE.¹⁵⁶ Moreover, no sex-specific change in receptor or ligand expression was noted with EAE progression in the spinal cord. *Gas6* and *Tyro3* genes are down-regulated during EAE while *Axl*, *Mertk* and *ProS* are all significantly upregulated, but not in a sex-specific manner. Could peripheral differences in mononuclear expression of *Axl* account for the mirrored relationship in EAE severity noted in this work? Higher basal levels of *Axl* might create a bias towards an anti-inflammatory milieu so that *Mertk* deficiency in female mice does not manifest with more severe disease in contrast to male mice, as proposed in Chapter 3. On the basis of the available evidence, it is not clear that resting or activated TAM receptor or ligand expression can account for the clinical observations in Chapters 4 and 5.

Importantly, conditional knock-out models like the one employed in this study result in gene deletion throughout immune maturation and development. This is an important limitation, as central inflammatory demyelination in humans is typically an acquired condition that manifests in adulthood. The establishment of central tolerance during immune development using cKO models could represent an important confounder that can be overcome by the use of inducible Cre-driver models, for example following tamoxifen exposure.

The importance of developmental (as opposed to acquired) immune phenotypes has been elegantly demonstrated in models of MERTK abrogation in the investigation of Type 1 diabetes in non-obese diabetic (NOD) mice. These mice exhibit several defects in the induction and/or maintenance of immune self-tolerance. As a result, autoreactive T cells mediate destruction of insulin-producing β cells within pancreatic islets. Autoimmune Type 1 diabetes subsequently emerges in these animals. *Mertk*^{-/-} NOD mice, however, are resistant to this process. They demonstrate lower levels of monocyte infiltration within pancreatic islets and IFN γ -producing CD4⁺ and CD8⁺ T cell counts are reduced. Importantly, the protective effect of *Mertk* deletion is due to enhanced thymic negative selection of autoreactive CD4 and CD8 precursor cells during immune maturation.¹⁷⁹ Contrast this result with the outcomes of small molecule MERTK blockade in non-cKO NOD mice. Within 24 hours of MERTK inhibition, islet-antigen specific CD4⁺ and CD8⁺ T cells experience arrested motility within pancreatic islets and CD8⁺ T cell activation is increased. The rapid onset of Type 1 diabetes follows shortly thereafter.¹⁸⁰ These data indicate that developmental or acquired deficiencies in TAM receptor signalling can result in markedly different phenotypic outcomes. The use of an inducible model of *Mertk* deletion to investigate changes in EAE may be required to more fully evaluate the results set out in this chapter. Moreover, inducible models of *Mertk* deficiency may more closely represent disease pathogenesis in human subjects, where the adult onset of multiple sclerosis after secondary sexual maturation is typical.

Taken together, the findings in this chapter suggest a key role for *Mertk* in the modulation of peripheral immune responses to central inflammatory demyelination. This work confirms that changes in *Mertk* expression may result in EAE exacerbation. While deletion of *Mertk* from CD11c⁺ cells does not cause alterations in immune constitution under physiologic conditions, upon EAE induction, increases in all three TAM receptors were observed. Further investigations concerned with sexually dimorphic responses to EAE are presented in the proceeding chapter.

CHAPTER 5: RESULTS

SEXUALLY DIMORPHIC RESPONSES TO INFLAMMATORY DEMYELINATING DISEASE

5.1 Introduction

Sexually dimorphic responses to infection,¹⁸¹ malignancy¹⁸² and autoimmunity¹⁸³ are well described in human disease. Adaptive and innate immune responses are generally enhanced in women, with higher levels of circulating immunoglobulins and CD4⁺ T cells identified in female patients. Females mount more robust responses to infection with increased cytokine production and they experience higher rates of tumour and allograft rejection.¹⁸⁴ Females are heavily overrepresented in a broad range of autoimmune disease; they are seven times more likely to experience systemic lupus erythematosus, Grave's disease, Sjogren's syndrome or Hashimoto's thyroiditis and twice as likely to be diagnosed with rheumatoid arthritis or scleroderma.¹⁸⁵ The relapsing-remitting form of multiple sclerosis is up to four times more common in females¹⁴ and epidemiological studies demonstrate that this female predominance is increasing over time.¹²

As noted in chapter 1, sex hormones account for at least part of this female preponderance and have been extensively studied.¹⁸⁶⁻¹⁸⁸ Apart from demonstrable effects on cell differentiation, cytokine profile and epigenetic alteration, sex hormones are further implicated in the development of autoimmune diseases by their tendency to manifest after pubertal sex differentiation has occurred.¹⁸⁹ Indeed, paediatric forms of MS do not demonstrate the sexual dimorphism that is apparent in adult relapsing-remitting disease.¹⁵ However, sex hormones alone do not account for the entire disparity in autoimmune disease burden identified in human subjects. Rates of SLE, RA and Sjogren's syndrome remain elevated in post-menopausal females compared to age-matched male controls.¹⁸⁹

In addition to sex hormones, certain immune traits in male and female mammals are sex-chromosome encoded. For example, the Toll-like receptor 7 (*TLR7*) gene is located on the X chromosome and may escape X inactivation in females, resulting in higher expression levels than in males.¹⁹⁰ *In vitro* exposure of peripheral blood mononuclear cells (PBMCs) to TLR7 ligands causes greater interferon- α (IFN α) production in females than males and plasmacytoid dendritic cells from both female humans and mice have higher IFN α levels following TLR7 ligand stimulation.¹⁹¹ Conversely, stimulation of dendritic cells with TLR9 ligands does not result in a sexually dimorphic response.¹⁹² There is also clearly interplay between sex hormones and sex chromosomes. Klinefelter's syndrome occurs when males carry an extra X chromosome (XXY) and results in low testosterone, increased gonadotrophins and elevated oestrogen concentrations. Immunologically, males with Klinefelter's syndrome respond to exogenous stimuli in a similar manner to females, with higher immunoglobulin levels, expanded CD4⁺ T cell populations and higher CD4/CD8 T cell ratios. These men also experience female-predominant autoimmune diseases at much higher rates than XY males. Interestingly, the immunological effects of Klinefelter's syndrome are reversed by testosterone therapy, indicating that sex hormones modulate immune responses in concert with sex chromosomes.¹⁹³

In the context of this evolving field of interest, it was intriguing to identify sexually dimorphic responses to EAE induction in multiple mouse cohorts, regardless of genotype. This chapter details an analysis of EAE outcomes in mice from multiple genotypes with a focus on sex differences.

The original EAE experiments involving wild-type C57BL/6 animals were performed by laboratory colleagues Michele Binder and Dr. Gerry Ma, in the context of different investigations. In light of the sexual dimorphism noted in CD11c-cre *Mertk*^{fl/wt} cohorts, I re-examined their basic experimental data in a bid to identify sexually dimorphic outcomes either in EAE severity or immunohistochemical analysis and to recast their

results in light of the emerging weight of evidence that TAM signalling is implicated in the development of central inflammatory demyelination.

5.2 Male Mice Demonstrate More Severe EAE Phenotype Compared to Female Littermates, Regardless of Genotype.

Anecdotally, I noted that male mice developed symptoms of EAE more rapidly than female mice and experienced a more severe clinical course, regardless of genotype. Male mice were more likely to be killed before the end of observation period due to severe disease and subsequent ethics committee requirements. When all CD11c-Cre *Mertk* floxed mice were stratified by sex, a striking and statistically significant difference in clinical outcomes was noted. Enhanced disease was observed in male mice, regardless of genotype, from days 11 to 18 post-induction with EAE ($p < 0.004$ for all times points) (**Fig. 5.1a**). At 15 dpi, the median grade in male mice was 2.25 compared to a median grade of 1.0 in female mice ($p = 0.0001$). When stratified by gender and genotype, male mice demonstrated more severe disease compared to female littermates (**Fig. 5.1b**). Survival analysis also confirmed earlier incidence of EAE in male mice regardless of genotype (Hazard ratio 0.53, 95% CI 0.36 – 0.77, $p = 0.0008$) (**Fig. 5.1c**).

A similar analysis was completed in CD11c-Cre *Mertk* floxed mice bred on a mixed background from 129/SvEv and C57BL/6 lineages. Again, a striking difference in clinical outcomes was noted between male and female mice, regardless of genotype. Significantly enhanced disease was observed in male mice from days 10 to 30 post-induction with EAE ($p < 0.003$ for all times points) (**Fig. 5.2a**). At 15 dpi, the median grade in male mice was 3.5 compared to a median grade of 2.25 in female mice ($p = 0.001$), indicating that while many male mice experienced very severe disease requiring early experiment termination, a substantially greater proportion of female mice were able to recover from this time point typically associated with disease peak. When stratified by gender and genotype, male mice demonstrated more severe disease compared to female littermates (**Fig. 5.2b**) Survival analysis confirmed greater incidence of EAE in male mice regardless of genotype (Hazard ratio 0.40, 95% CI 0.21 – 0.78, $p = 0.003$) (**Fig. 5.2c**).

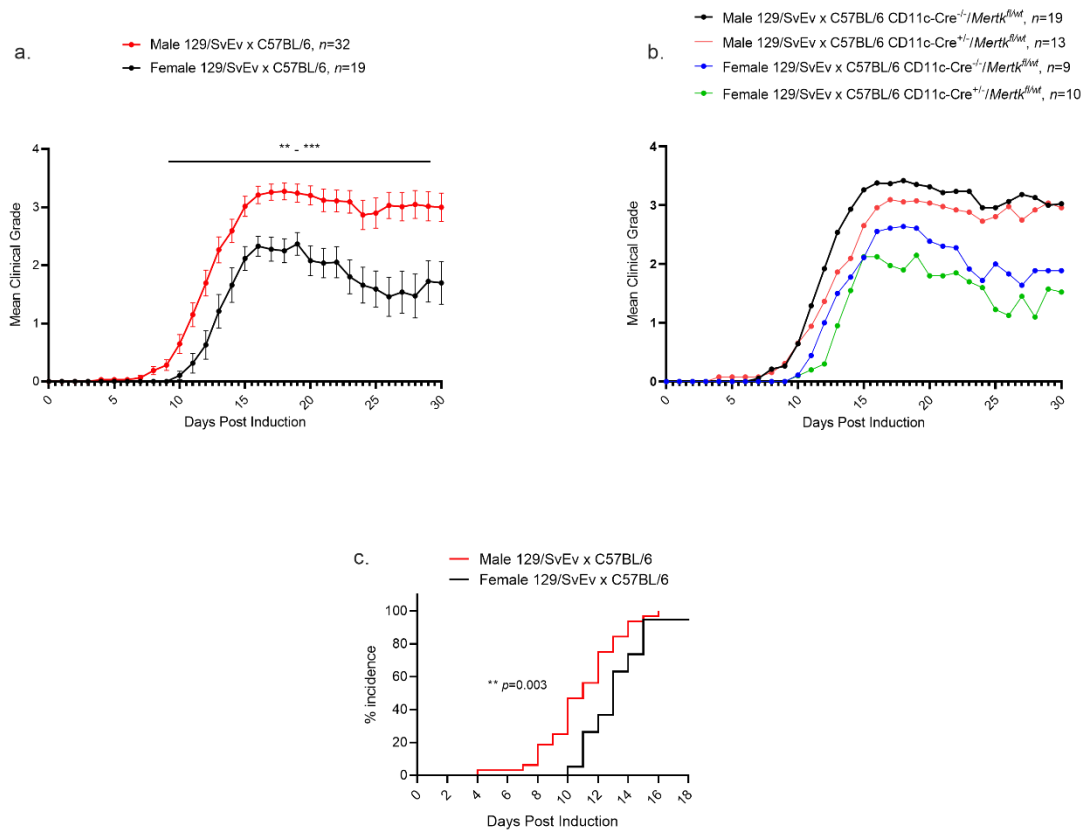


Figure 5.2. Male mice on mixed 129/SvEv x C57BL/6 background demonstrate more severe EAE phenotype compared to female littermates. **a.** *Mertk* floxed animals on mixed 129/SvEv and C57BL/6 background stratified by sex without reference to genotype demonstrate significantly enhanced disease in male mice from days 10 to 30 post-induction with EAE (Mann-Whitney U test, $^{**}p < 0.003$ for all times points); **b.** Mean clinical grades for male and female CD11c-Cre^{+/-}/*Mertk*^{fl/wt} and CD11c-Cre^{-/-}/*Mertk*^{fl/wt} mice on mixed 129/SvEv and C57BL/6 background **c.** Survival analysis demonstrates earlier incidence of EAE in male mice on mixed 129/SvEv and C57BL/6 background regardless of genotype (Hazard ratio 0.40, 95% CI 0.21 – 0.78, $^{**}p = 0.003$, Log-rank (Mantel-Cox) test).

Finally, clinical outcomes in EAE were assessed in wild-type C57BL/6 male and female mice. Again, significantly more severe disease was identified in male mice compared to female littermates. Enhanced disease was observed in male mice, from days 11 to 13 post-induction with EAE ($p < 0.03$ for all times points). (**Fig. 5.3a**). At 13 dpi, the median grade in male mice was 2.0 compared to a median grade of 0 in female mice ($p = 0.005$). Survival analysis did not indicate a difference in timing of disease onset. (Hazard ratio 0.64, 95% CI 0.31 – 1.32) (**Fig. 5.3b**).

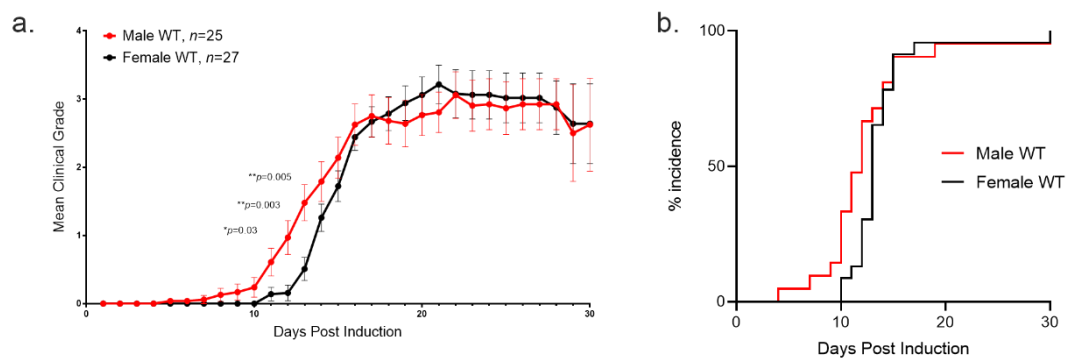


Figure 5.3. Male WT mice demonstrate more severe EAE phenotype compared to female littermates. a. C57BL/6 mice stratified by sex demonstrate significantly enhanced disease in male mice from 11 to 13 dpi (Mann-Whitney U test, $^{*}p<0.03$ for all times points); **b.** Survival analysis demonstrates trend towards earlier disease onset in male mice (Hazard ratio 0.64, 95% CI 0.31 – 1.32).

5.3 Wild-Type C57BL/6 Female Mice Demonstrate Different Cellular Responses to EAE Compared to Male Littermates

Based on these clinical observations, immunohistochemical analysis of the lumbar spinal cord of wild-type C57BL/6 mice was then undertaken at days 8, 15 and 21 dpi, to examine potential differences in cellular responses in the effector stage of EAE and also at or around disease peak. Unchallenged mice were used as negative controls. Myelination, oligodendrocyte and microglial responses were assessed with the dorsal column of the spinal cord.

To assess oligodendrocyte lineages, separate markers to label oligodendrocyte precursor cells (OPCs)(α -type platelet-derived growth factor receptor (PDGFR α)) and all oligodendrocytes (Olig2) were employed. A significant difference in the number of Olig2⁺ cells was identified at 21 dpi in the lumbar spinal cord of female wild-type C57BL/6 mice compared to male littermates ($p=0.009$) (**Fig. 5.4a**). No significant difference was noted in the number of PDGFR α ⁺ cells at any time point ($p>0.05$) (**Fig. 5.4b**).

To assess microglial responses to EAE, the marker ionised calcium binding adapter molecule 1 (IBA1) was employed. Female wild-type C57BL/6 mice demonstrated

significantly increased levels of IBA1 in the lumbar spinal cord at 8 dpi ($p=0.003$), during the effector stage of EAE (**Fig. 5.4c**).

Myelination was assessed by immunofluorescent staining using the RIP antibody which has avidity for the myelin marker 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNPase).¹⁹⁴ No significant difference was observed in the mean fluorescent intensity of RIP staining between male and female wild-type C57BL/6 mice at any time point ($p>0.05$) (**Fig. 5.4d**).

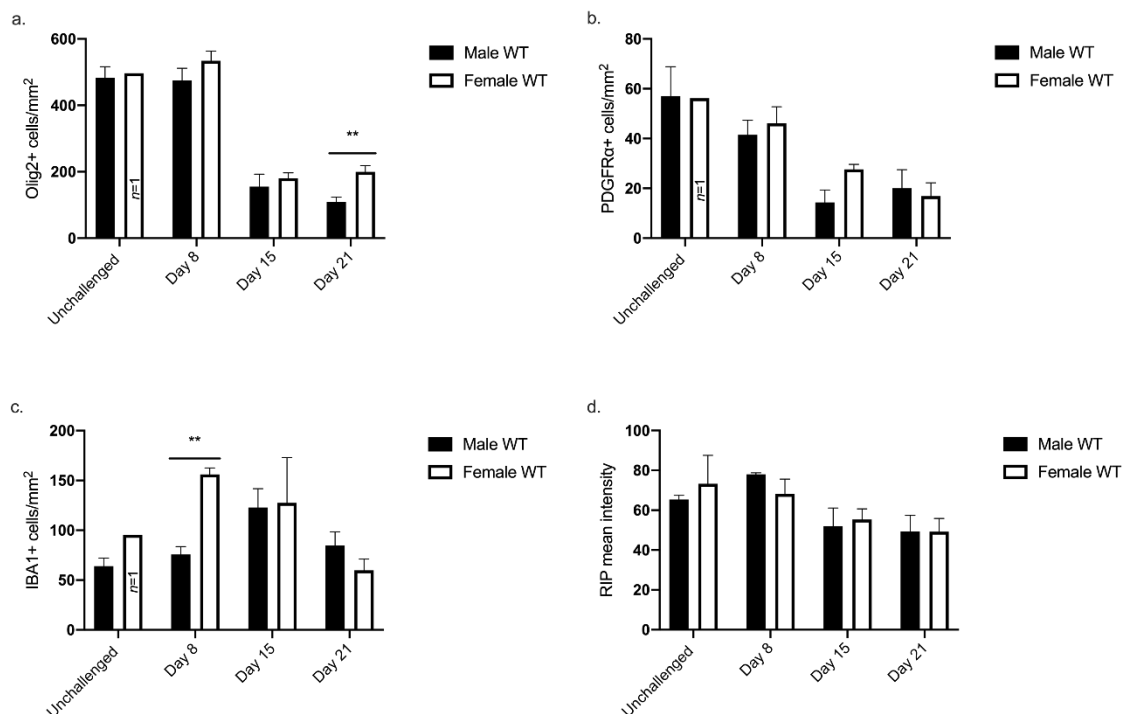


Figure 5.4. Wild-type C57BL/6 female mice demonstrate different cellular responses to EAE compared to male littermates. **a.** Significant difference in the number of Olig2+ cells at 21 dpi in the lumbar spinal cord of female wild-type C57BL/6 mice compared to male littermates (Student's T-test, $**p=0.009$); **b.** No significant difference in the number of PDGFRα+ cells in male and female wild-type C57BL/6 mice at any time point; **c.** Significant difference in the number of IBA1+ cells at 8 dpi in the lumbar spinal cord of female wild-type C57BL/6 mice compared to male littermates (Student's T-test, $**p=0.003$); **d.** No significant difference in mean fluorescence intensity of RIP staining in male and female wild-type C57BL/6 mice at any time point.

5.4 Discussion

Sexual dimorphism in EAE outcomes was first described in rats in 1978.¹⁹⁵ Female Lewis rats immunised with CNS tissue demonstrated a relapsing form of central demyelination while male rats experienced a monophasic illness. A sex-hormone phenomenon related to the oestrus cycle was proposed. Since that time, sexually-dependent variability in EAE outcomes have been identified depending on animal strain,¹⁹⁶ immunological substrate,¹⁹⁷ induction protocol,¹⁹⁸ animal age¹⁹⁹ and even seasonality.²⁰⁰ Within *Mus musculus*, particular strains have been identified where females experience more severe disease (SJL), where males experience more severe disease (NZW/LACJ) or where clinical outcomes are reportedly evenly matched (C56BL/6).¹⁹⁶

In keeping with pathology studies in humans, sex hormones and sex chromosomes have been implicated in these differential outcomes. While it has long been established that pregnancy²⁰¹ and the provision of exogenous oestrogens^{20, 175, 176} can ameliorate the outcome of EAE in female mice, very recent work has identified sex hormone differences in the spinal cords of male and female rats, related to clinical outcomes. Specifically, male Dark Agouti (DA) rats demonstrated a marked decline in spinal cord pregnenolone (the first steroidogenic product) during the acute phase of EAE (14 dpi) while in female DA rats a similar decline was not seen until the chronic phase of EAE was established (45 dpi). No differences were noted in the plasma of the EAE-treated rats and a trend towards early onset and more severe disease was seen in male rats. This suggests that disease outcomes are modulated in the target organ itself, rather than the periphery.¹⁹⁷

Sex chromosomes have also demonstrated a direct effect on EAE outcomes. While young male SJL mice (≤ 8 weeks) are relatively resistant to EAE, consomic young SJL males carrying the B10.S Y chromosome (common to B10.PL and C57BL/6 mice) experience significantly more severe disease, equivalent to observations in female SJL mice or in adult SJL males.²⁰⁰ The authors of this report hypothesise that polymorphisms in the *Sry* gene account for the alteration in EAE severity and confer on young male

SLJ.Y^{B10.S} mice the ability to process and present antigen that is not typically present before adulthood.

Finally, environment and exposure also modify immune outcomes in EAE, in parallel with experiences in human disease. Very recent work has demonstrated sexually dimorphic outcomes in MOG₃₅₋₅₅ EAE in C57BL/6 mice following *ad libitum* provision of moderate quantities of alcohol prior to EAE induction. Alcohol-fed males experienced markedly attenuated EAE scores compared to alcohol-fed females and control-fed male littermates. In the spinal cord, lower microglial density was noted in ameliorated males while in the gut sex-specific alterations in microbiota were identified with significant enrichment of Clostridial taxa known to be associated with protection against EAE.²⁰²

Most importantly for the work set out in this chapter, an analysis of the control-fed male and female C57BL/6 animals in this last study also identified early onset of EAE symptoms in male mice with significantly more severe clinical scores during the effector stage of EAE compared to female controls. Others have also reported similar trends towards more severe disease in male C57BL/6 mice,²⁰³ supporting the striking finding here that multiple cohorts of male C57BL/6 mice are more severely affected by EAE, regardless of other genetic modifications. Finally, reviewing a frequently cited report that C57BL/6 mice experience no sex-specific differences in outcome reveals relatively a smaller number of animals ($n=12$ per sex)¹⁹⁶ compared to the numbers reported herein and in other published reports.

Accordingly, sex-specific differences in the response of adult C57BL/6 mice to EAE induction can be predicted in future experiments. While it was outside the scope of this work to elucidate the specific mechanisms of sexually dimorphic outcomes in EAE (given a focus instead on changes in outcome based on TAM receptor signalling), repeat EAE experiments in oophorectomized and sham surgery female cohorts would test the hypothesis that sex hormones are key to the variation in clinical outcomes. Spinal cord and adjacent lymph node analysis of immune cell constituents and levels of associated

pro- and anti-inflammatory cytokines should allow for specific characterisation of mechanistic differences driving altered clinical outcomes, as well as the relevant tissue location contributing to EAE severity.

The histological data available for correlation with clinical outcomes were limited in this series. However, point differences in the number of IBA1^{+ve} microglia/macrophages were noted at 8 dpi with increased numbers in the less affected, female mice. Similarly, a single point increase in the number of oligodendrocytes was also noted in females at 21 dpi. On initial review, these results seem inconsistent. IBA1 is a calcium binding protein required for ruffling of the microglia/macrophage membrane, a typical feature of cell activation.²⁰⁴ The presence of microglial activation is typically associated with more severe clinical outcomes²⁰³ and higher numbers in female WT C57BL/6 during the effector phase of EAE should connote more severe disease. An opposite trend is observed in these data. An alternate explanation, however, may rest on the hypothesis that increased IBA1^{+ve} microglia/macrophages numbers in female mice represent a reparative as opposed to a pathogenic response. In that setting, increased numbers of oligodendrocytes might be predicted, as a response towards remyelination is stimulated. Alternatively, increased IBA1 staining may reflect greater density of microglia as opposed to an underlying change in activation state. This may be anti-inflammatory or reparative in nature, for example through the phagocytosis of cell debris. Clearly, further analysis is required in WT C57BL/6 animals to explain the relationship between microglial and oligodendrocyte numbers identified herein.

As noted in Chapter 4, sex-specific differences in inflammatory infiltrates were not identified in the lumbar spinal cords of *Mertk* deficient cohorts following EAE induction. Lower mononuclear activation in the setting of higher endogenous *Axl* levels in female mice may create an environment in which the anti-inflammatory effects of increased oestrogens are able to negatively regulate EAE severity. In effect, the presence of female sex hormones, which are known to encourage differentiation of regulatory B cells^{205, 206}

and M1-to-M2 microglial switching,^{207, 208} may allow the more clinically restrained phenotype to become penetrant in female mice.

Taken together, the data presented here (and in Chapter 4) offer intriguing suggestions of a key role for TAM receptors and their ligands along with sex in the modulation of inflammatory demyelination. It will be essential to explore key interactions between the immune responses modulated by these receptors and the totality of the immune environment created by sex hormones, sex chromosomes and environmental exposures. It is possible that modulation of TAM receptor functions in the treatment of human disease may itself require a sex-specific approach to patient selection.

CHAPTER 6: RESULTS

MERTK EXPRESSION BY HUMAN PERIPHERAL BLOOD MONOCYTES

6.1 Introduction

MERTK is a member of the TAM (TYRO3, AXL and MERTK) family of receptor tyrosine kinases. As noted in Chapter 1, the TAMs are part of a dynamic, compensatory system that responds to inflammatory insults. TAM receptor expression by innate immune cells is upregulated in response to phagocytosis, in part via activation of the retinoic acid pathway.²⁰⁹ Of relevance to the work set out in this thesis, the ability of the TAMs and their cognate ligands to modify the outcomes of central demyelination has been demonstrated in numerous animal studies.^{103, 104, 106} In humans, *MERTK* has been identified as a susceptibility gene for MS and variants within the gene are expression quantitative trait loci (eQTLs) within innate immune cells in the peripheral blood.¹⁵³

The HLA genes encode the major histocompatibility complex (MHC) which is expressed by professional antigen presenting cells for the purpose of adaptive immune cell activation with processed antigen. Variations in HLA genotype influence this activity and HLA genes are associated with a broad range of autoimmune disease. For example, Caucasians heterozygous for HLA-DRB1*1501 within the major histocompatibility complex have an odds ratio for developing MS of approximately 3.0, representing the strongest susceptibility allele for MS.²⁴ Over 200 other susceptibility loci have been now identified via genome-wide association studies (GWAS) but each conveys an odds ratio of 1.2 or less.²³ In this context, it is notable that the predominant susceptibility alleles of *MERTK* for the majority of the MS population drive high expression of MERTK in monocytes. However, for individuals homozygous for HLA-DRB1*1501, itself a major susceptibility gene for MS, there is enhanced susceptibility and severity of the disease amongst those homozygous for low expressing MERTK alleles, specifically the polymorphism at rs7422195.¹⁵³ This stratification of risk and severity based on HLA-DRB1*1501 status suggests that *MERTK*'s contextual influence is mediated by antigen

presentation. In particular, increased presentation of myelin-related antigens might correspond with relapse activity during inflammatory attacks of MS.

Many studies have examined the sera and cerebrospinal fluid of patients with MS in a bid to identify a reliable biomarker that might correlate with disease activity. None has yet been found.²¹⁰ Examination of the cerebrospinal fluid of patients with MS for the intrathecal synthesis of oligoclonal bands is frequently performed and forms part of the 2017 revised McDonald Criteria for the diagnosis of that condition.⁴ However, CSF oligoclonal bands are not exclusive to MS²¹¹ and serial performance of lumbar punctures to assess disease activity would be invasive and potentially lead to complications. Accordingly, the identification of a serum biomarker to assist in the diagnosis and monitoring of MS activity could have substantial utility in clinical settings. The work set out in this chapter investigates whether levels of MERTK expressed by circulating monocytes and dendritic cells might have utility as an indicator of underlying MS activity.

6.2 Recruitment of Patients and Control Subjects

Patients with relapsing-remitting MS experiencing clinical relapse were recruited from the MS and Neuroimmunology Clinic at the Royal Melbourne Hospital. In order to prevent the recruitment of patients experiencing pseudo-relapse, all patients were examined by a consultant neurologist prior to study entry. Only patients experiencing a relapse of sufficient intensity, in the opinion of the examining neurologist, to justify treatment with high dose corticosteroids were recruited. Samples of PBMCs were isolated from these individuals and stored in liquid nitrogen until analysis for MERTK expression, as described in Chapter 2.

Patients recruited during relapse were recalled after a period of at least three months for a second blood collection so that PBMC expression of MERTK could be compared in relapse and remission. No eligibility restrictions were placed upon the concomitant use of disease modifying therapies for MS. However, patients who either changed

medication between collections or received second courses of alemtuzumab or cladribine were excluded from the study.

Healthy human controls were also recruited and blood collection and PBMC isolation was also performed at study entry and after a period of at least three months from these individuals.

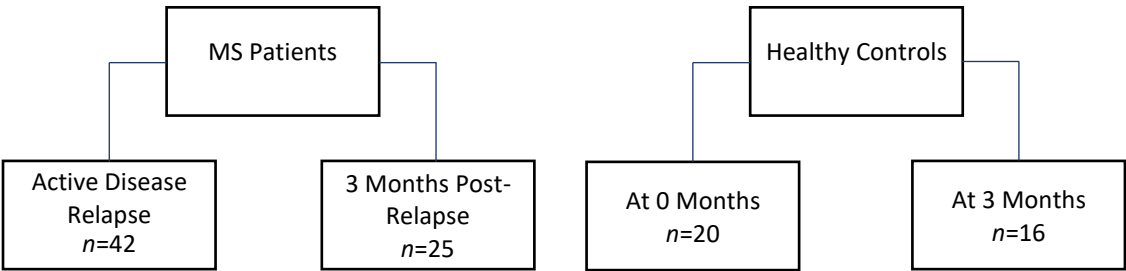


Figure 6.1. Recruitment for PBMC Analysis. Recruitment of patients with relapsing-remitting MS and health controls for analysis of monocyte and dendritic cell expression of MERTK.

Forty-two patients experiencing an MS relapse of sufficient intensity to warrant steroid administration were identified and recruited. Among those 42 patients, 25 returned after an interval of at least three months at a time when relapse-related symptoms were not reported for repeat sample collection. Twenty healthy controls were recruited and 16 of those subjects returned for repeat sample collection.

Demographic information regarding patients and control subjects who completed Time 0 and Time 3 blood collections is set out in Table 6.1. In both the MS patient and control subject groups one participant could not be genotyped for the *MERTK* polymorphism at rs7422195. Those subjects were excluded from further analysis.

Table 6.1: MS Patient and Control Subject Demographic Information

	MS Patients (n=25)	Control Subjects (n=16)
Age (Years)	37 ± 7.2	30.5 ± 9.4
Sex (F:M)	21:4	11:5
Ethnicity		
Caucasian	21	14
Asian	1	2
MEA	2	0
Hispanic	1	0
Genotype at rs7422195		
GG	7	5
GA	14	7
AA	3	3

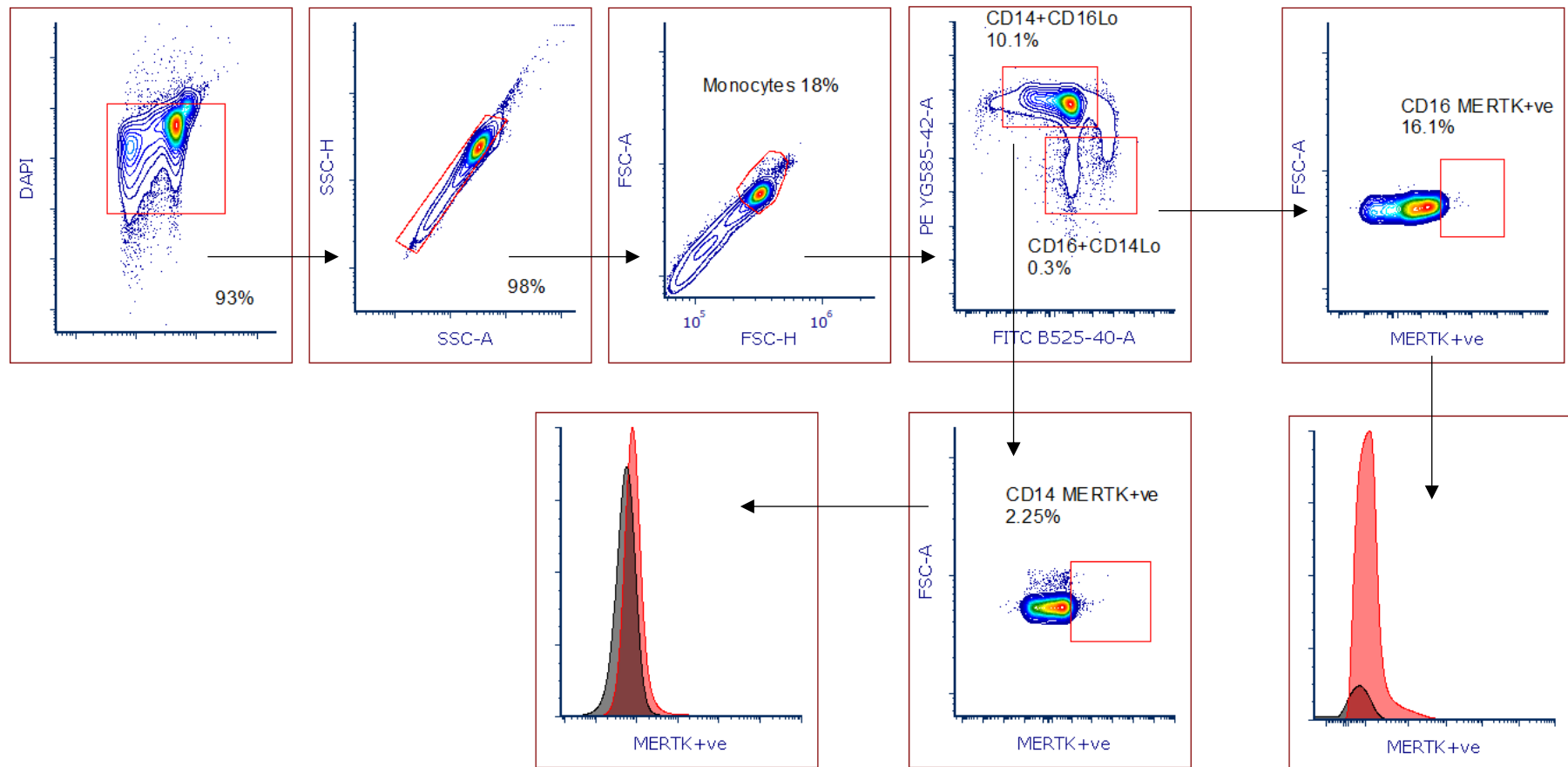
6.3 Monocyte Expression of MERTK in MS Relapse and Remission

Classically activated monocytes were identified by high levels of CD14 (a co-receptor for Toll-like receptor 4, mediating lipopolysaccharide signalling) and low levels of CD16 (Fc gamma receptor IIIa). Non-classical monocytes were identified by low levels of CD14 and high levels of CD16.¹⁴⁹ The flow cytometry gating strategy employed to identify these cells is set out in **Figure 6.1a**. No statistically significant differences were identified in MERTK expression during relapse and remission in MS patients by median fluorescent intensity (MFI)(**Fig. 6.4b,d**), the number of MERTK positive events per sample (**Fig. 6.4f,h**) or the proportion of MERTK positive classical or non-classical monocytes (**Fig 6.4 j,l**).

No statistically significant differences were identified in MERTK expression in healthy controls at study entry or after a period of at least three months by median fluorescent intensity (MFI)(**Fig. 6.4c,e**), the number of MERTK positive events per sample (**Fig. 6.4g,i**) or the proportion of MERTK positive classical or non-classical monocytes (**Fig. 6.4 k,m**). In addition, no differences were seen in MERTK expression *between* MS patients and control subjects at either time point. In both MS patients and healthy controls, the non-

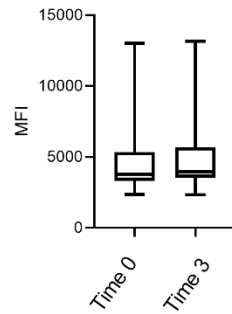
classical monocyte populations demonstrated an increase in the proportion of cells expressing MERTK.

a.

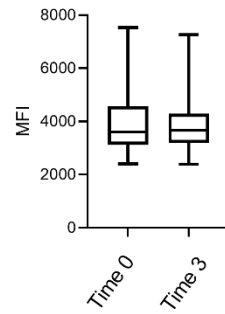


Median Fluorescent Intensity

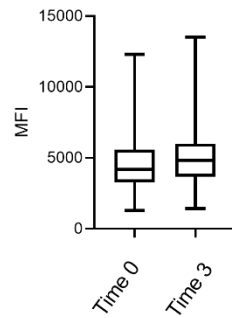
b. MS Patients - CD14



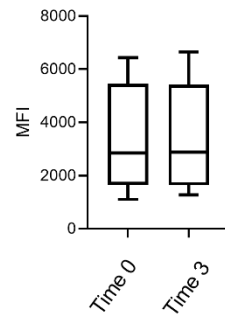
c. Healthy Controls - CD14



d. MS Patients - CD16

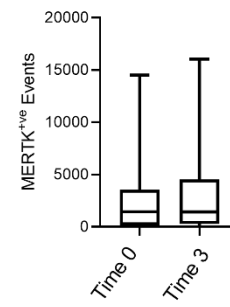


e. Healthy Controls - CD16

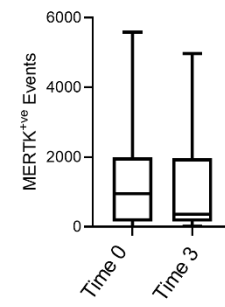


No. MERTK⁺ Events

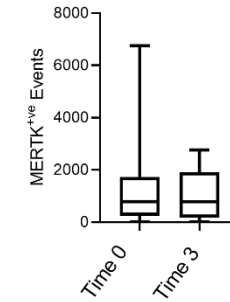
f. MS Patients - CD14



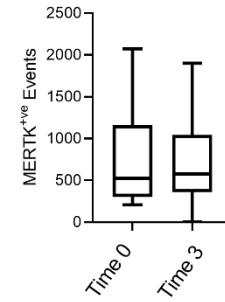
g. Healthy Controls - CD14



h. MS Patients - CD16



i. Healthy Controls - CD16



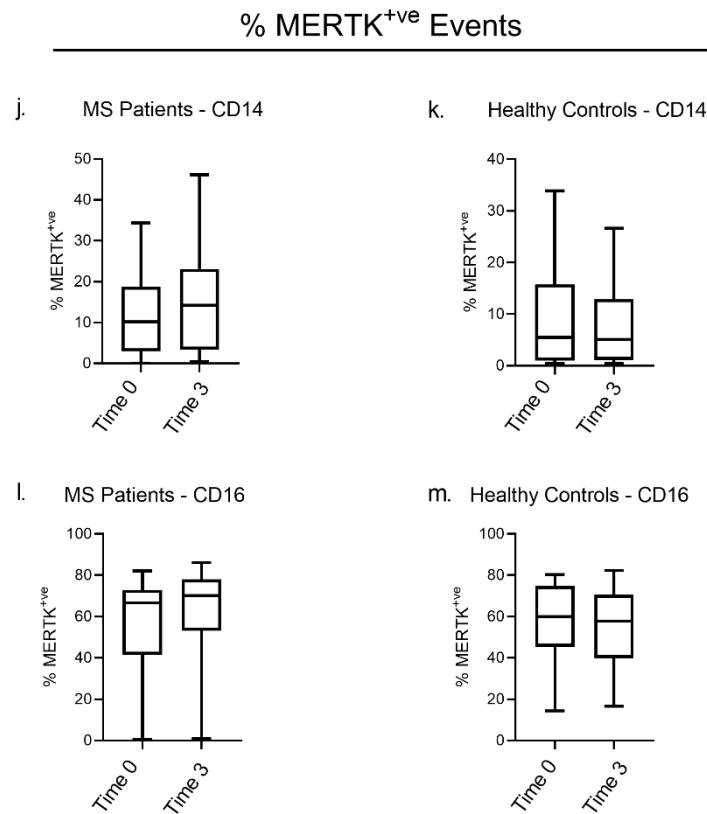
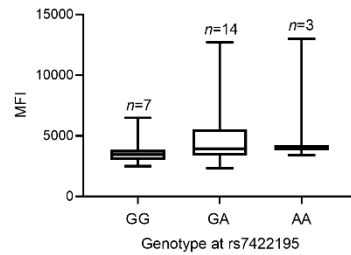


Figure 6.2. No difference in MERTK expression in CD14⁺CD16^{LO} or CD16⁺CD14^{LO} monocytes during MS relapse or remission. **a.** Gating strategy for the identification of MERTK⁺ CD14⁺CD16^{LO} and CD16⁺CD14^{LO} monocytes; **b.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **c.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3); **d.** Median fluorescent intensity of MERTK⁺ cells in CD16⁺CD14^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **e.** Median fluorescent intensity of MERTK⁺ cells in CD16⁺CD14^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3); **f.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **g.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3); **h.** Number of MERTK⁺ cells in CD16⁺CD14^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **i.** Number of MERTK⁺ events in CD16⁺CD14^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3); **j.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **k.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3); **l.** Percentage of MERTK⁺ events in CD16⁺CD14^{LO} monocytes in MS patients at study entry (Time 0) and after a period of a least three months (Time 3); **m.** Percentage of MERTK⁺ events in CD16⁺CD14^{LO} monocytes in healthy controls at study entry (Time 0) and after a period of a least three months (Time 3);

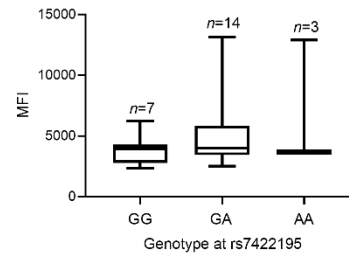
In order to assess for potential genotype-specific responses to events of active inflammatory demyelination, these monocyte expression data were stratified by the *MERTK* polymorphism at rs7422195. As previously noted, the low-expressing GG allele is associated with increased MS susceptibility and severity in patients homozygous for HLA-DRB1*1501. In distinction to published results from laboratory colleagues,¹⁵³ no genotype-specific differences were seen in *MERTK* expression in the classical monocyte population during MS relapse or remission by analysis of MFI (**Fig. 6.3a-d**), number of *MERTK*⁺ cells (**Fig. 6.3e-h**) or the percentage of *MERTK*⁺ cells (**Fig. 6.3i-l**).

Median Fluorescent Intensity - CD14⁺ve

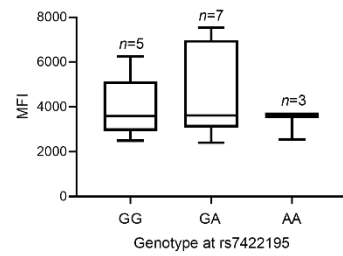
a. MS Patients - Time 0



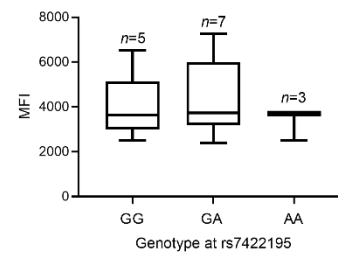
b. MS Patients - Time 3



c. Healthy Controls - Time 0

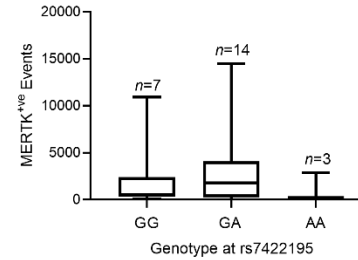


d. Healthy Controls - Time 3

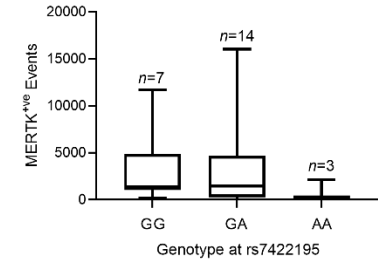


No. MERTK⁺ve Events - CD14⁺ve

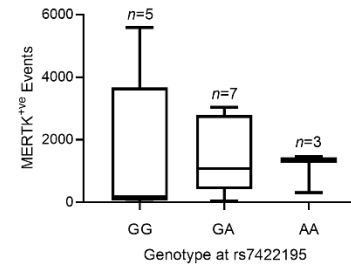
e. MS Patients - Time 0



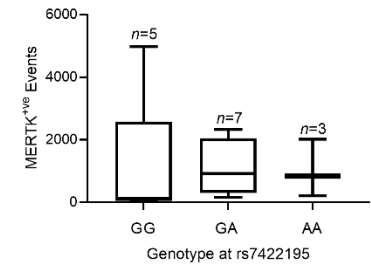
f. MS Patients - Time 3



g. Healthy Controls - Time 0



h. Healthy Controls - Time 3



% MERTK⁺ Events - CD14⁺

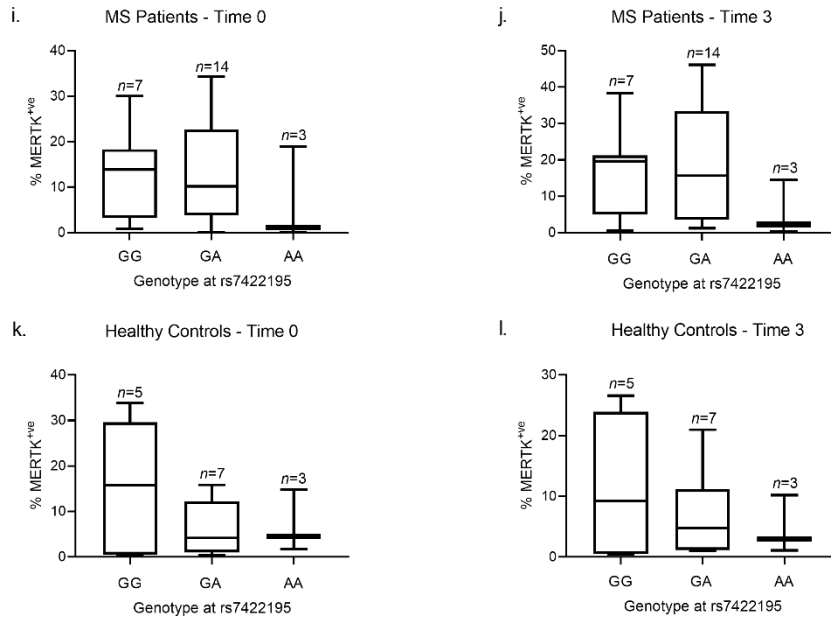
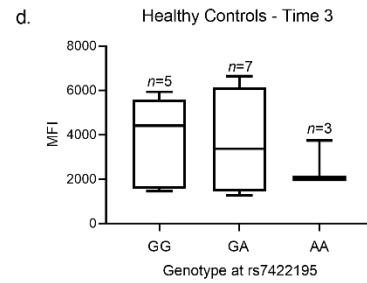
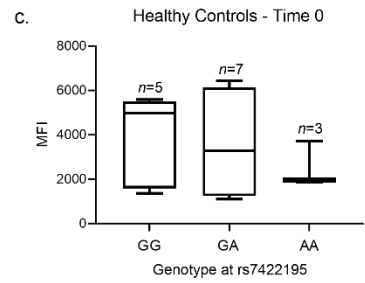
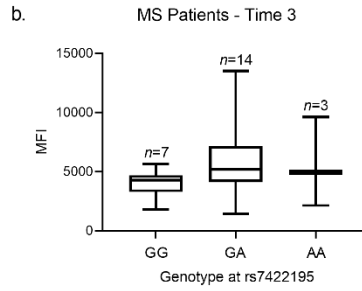
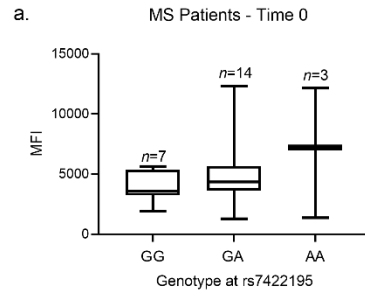


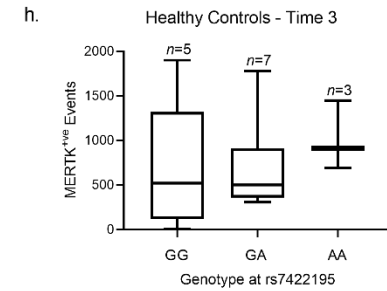
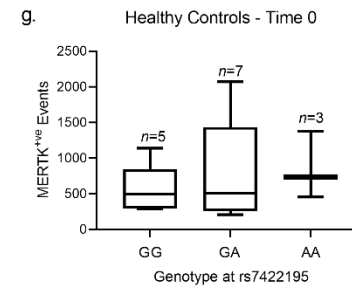
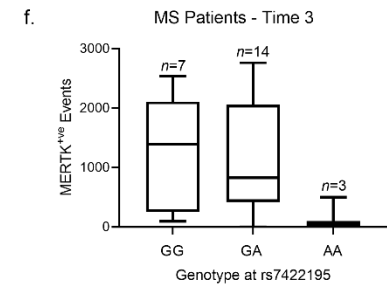
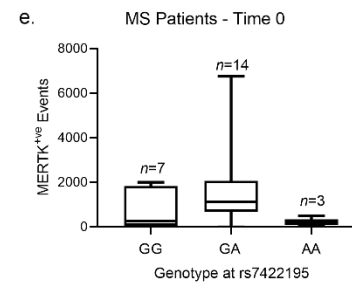
Figure 6.3. No difference in MERTK expression in CD14⁺CD16^{LO} monocytes when stratified by genotype at rs7422195 in MS relapse or remission. **a.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **b.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **c.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **d.** Median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195; **e.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **f.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **g.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **h.** Number of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195; **i.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **j.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **k.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **l.** Percentage of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195. One-way ANOVA with Tukey's multiple comparison correction, $p > 0.05$. Grouped expression data are plotted as Tukey box and whiskers.

CD16⁺CD14^{LO} non-classical monocytes were analysed in the same manner. Again, no genotype-specific differences were seen in MERTK expression in the non-classical monocyte population during MS relapse or remission by analysis of MFI (**Fig. 6.4a-d**), number of MERTK⁺ cells (**Fig. 6.4e-h**) or the percentage of MERTK⁺ cells (**Fig. 6.4i-l**).

Median Fluorescent Intensity - CD16⁺ve



No. MERTK⁺ve Events - CD16⁺ve



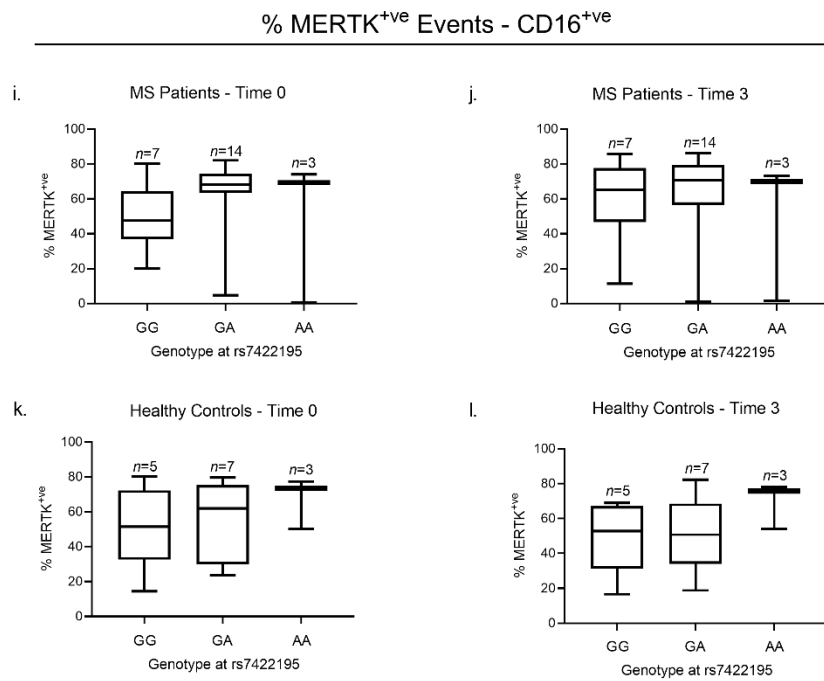
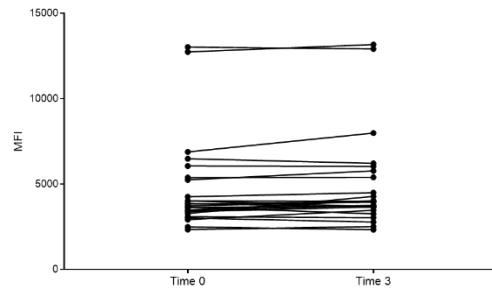


Figure 6.4. No difference in MERTK expression in CD16⁺CD14^{LO} monocytes when stratified by genotype at rs7422195 in MS relapse or remission. **a.** Median fluorescent intensity of MERTK⁺ cells in CD16⁺CD14^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **b.** Median fluorescent intensity of MERTK⁺ cells in in CD16⁺CD14^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **c.** Median fluorescent intensity of MERTK⁺ cells in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **d.** Median fluorescent intensity of MERTK⁺ cells in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195; **e.** Number of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **f.** Number of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **g.** Number of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **h.** Number of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195; **i.** Proportion of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in MS patients at Time 0, stratified by genotype at rs7422195; **j.** Proportion of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in MS patients at Time 3, stratified by genotype at rs7422195; **k.** Proportion of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 0, stratified by genotype at rs7422195; **l.** Proportion of MERTK⁺ events in in CD16⁺CD14^{LO} monocytes in healthy controls at Time 3, stratified by genotype at rs7422195. One-way ANOVA with Tukey's multiple comparison correction, $p > 0.05$. Grouped expression data are plotted as Tukey box and whiskers.

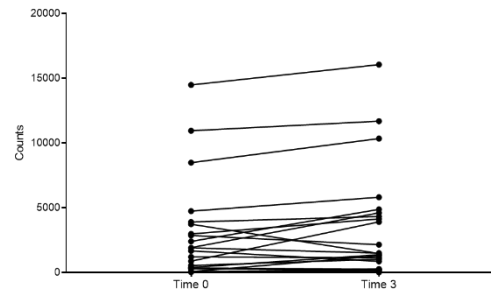
Finally, an analysis of each paired result was performed in a bid to identify potential sub-groups of patients that might have demonstrated disease-related changes in MERTK expression in classical and non-classical monocytes. No obvious trends emerged with regard to expression based on rs7422195 genotype in classical (**Fig. 6.5a-c**) or non-classical (**Fig. 6.5d-f**) monocytes in MS patients or controls subjects (not shown). In fact, in most instances the MFI, number of MERTK⁺ cells and proportion of MERTK⁺ cells were stable at both time points.

MS Patients - CD14⁺

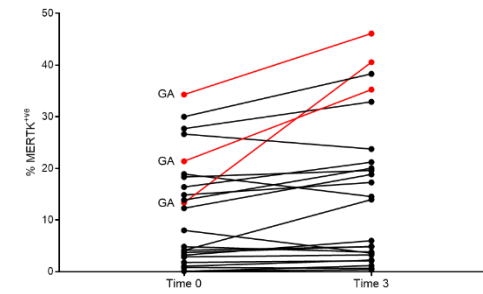
a. Median Fluorescent Intensity



b. No. MERTK⁺ Events

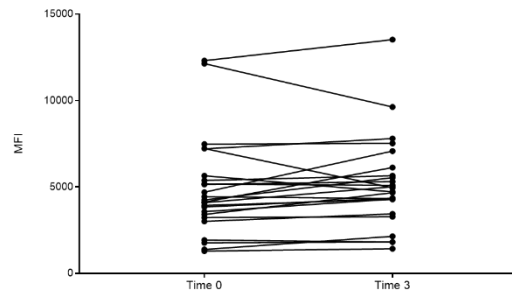


c. % MERTK⁺ Events

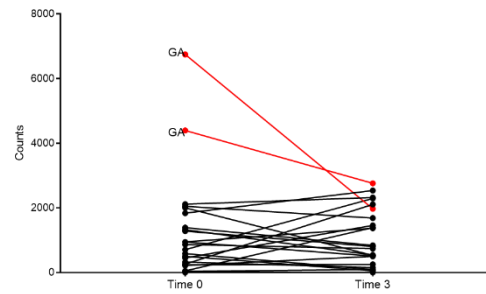


MS Patients - CD16⁺

d. Median Fluorescent Intensity



e. No. MERTK⁺ Events



f. % MERTK⁺ Events

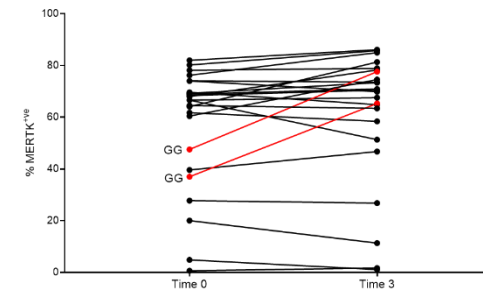


Figure 6.5. Analysis of paired results from each study participant did not identify relevant sub-groups based genotype at rs7422195. **a.** Paired results for median fluorescent intensity of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in MS patients at Time 0 and Time 3; **b.** Paired results for MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 0 and Time 3; **c.** Paired results for proportion of MERTK⁺ events in CD14⁺CD16^{LO} monocytes in MS patients at Time 0 and Time 3; **d.** Paired results for median fluorescent intensity of MERTK⁺ cells in CD16⁺CD14^{LO} monocytes in MS patients at Time 0 and Time 3; **e.** Paired results for MERTK⁺ events in CD16⁺CD14^{LO} monocytes in MS patients at Time 0 and Time 3; **f.** Paired results for proportion of MERTK⁺ events in CD16⁺CD14^{LO} monocytes in MS patients at Time 0 and Time 3; **g.** Paired results for proportion of MERTK⁺ cells in CD14⁺CD16^{LO} monocytes in controls subject at Time 0 and Time 3; **h.** Paired results for proportion of MERTK⁺ events in CD16⁺CD14^{LO} monocytes in control subjects at Time 0 and Time 3. Genotype at rs7422195 noted for selected paired results.

6.3.1 The Use of Fresh or Frozen Peripheral Blood Mononuclear Cells Does Not Alter Assessment of MERTK Expression by Flow Cytometric Analysis

As noted above, published work presented by laboratory colleagues indicated a genotype-specific difference in the expression of MERTK in CD14⁺ monocytes in MS patients during remission. In particular, it was noted that patients carrying the AA allele of the rs7422195 polymorphism demonstrated a statistically significant increase in the proportion of classical monocytes expressing MERTK compared to GA and GG genotypes.¹⁵³ Importantly, those data were ascertained through the analysis of fresh monocytes that underwent flow cytometric analysis on the day of collection. In order to control for potential differences in MERTK expression as a result of long-term liquid nitrogen storage, fresh and frozen monocyte expression analysis was performed using control subject samples with known polymorphisms at rs7422195. No difference in MERTK expression was identified between fresh and frozen monocyte samples in CD14⁺CD16^{Lo} monocytes in either genotype at rs7422195. Among CD16⁺ cells, higher proportions of MERTK⁺ cells were seen in both genotypes in cells exposed to a single freeze-thaw cycle (**Fig. 6.6a,b**).

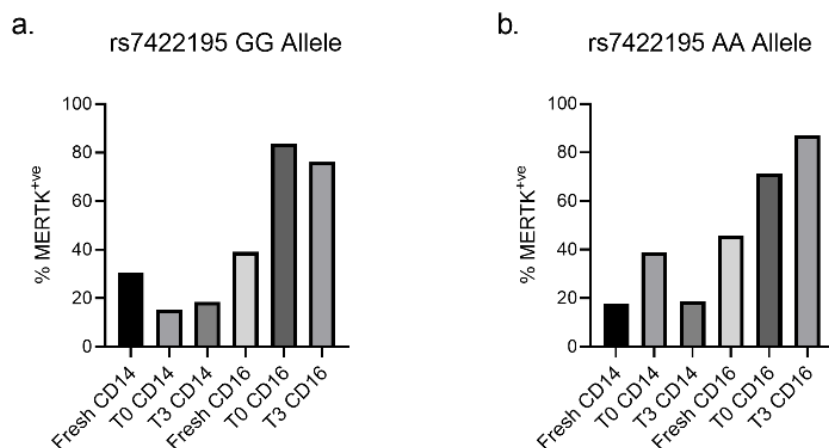


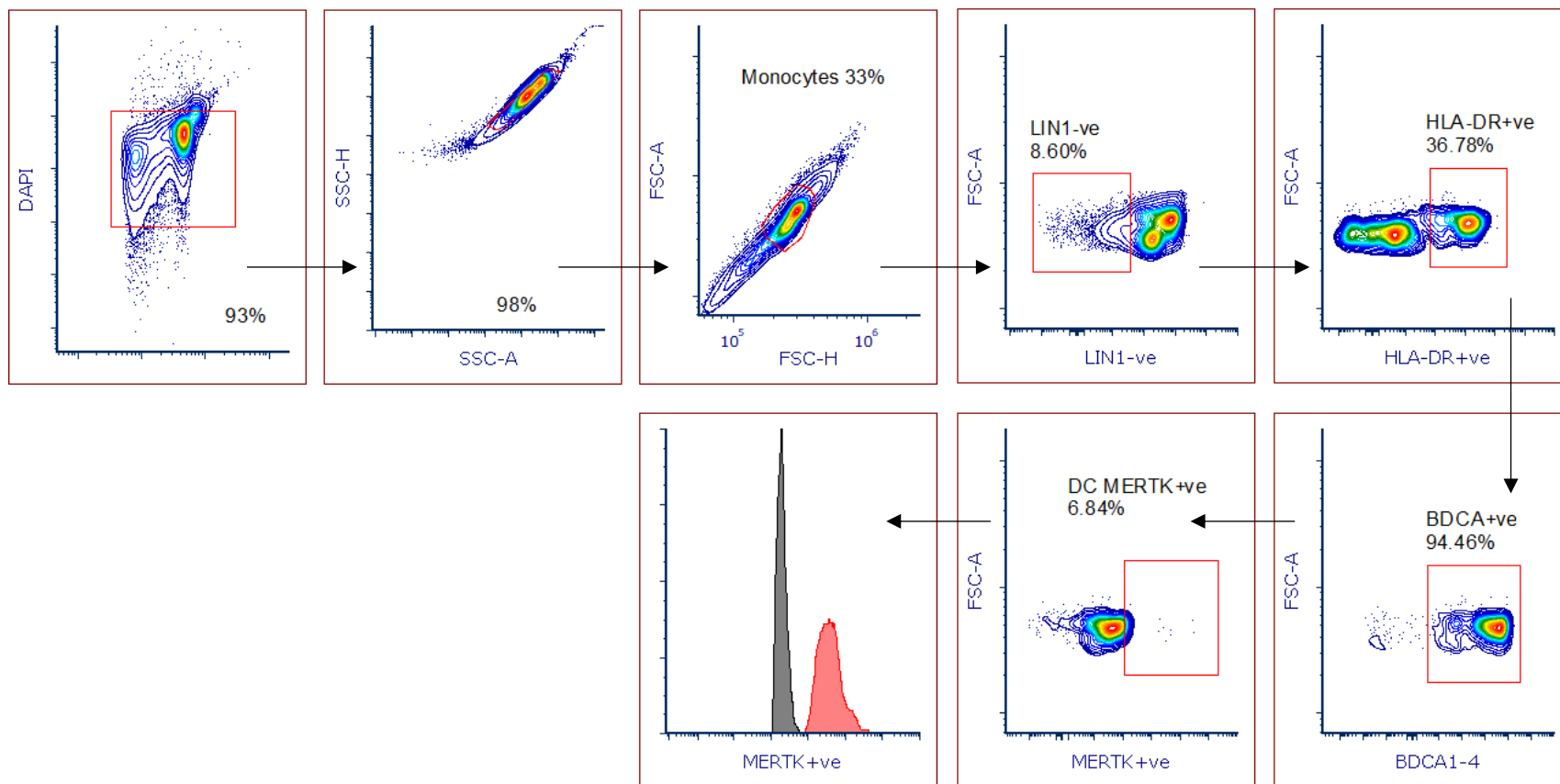
Figure 6.6. Comparison of MERTK Expression in Fresh and Frozen Monocyte Samples. **a.** Proportion of MERTK⁺ cells among CD14⁺CD16^{Lo} and CD16⁺CD14^{Lo} monocytes from fresh, Time 0 and Time 3 samples from a control subject with GG allele at rs7422195; **b.** MERTK expression by CD14⁺CD16^{Lo} and CD16⁺CD14^{Lo} monocytes from fresh, Time 0 and Time 3 samples from a control subject with AA allele at rs7422195.

6.4 Dendritic Cell Expression of MERTK in MS Relapse and Remission

Dendritic cells were identified by first applying a LIN^{-ve} marker to the PBMC population to exclude CD3, CD14, CD16, CD19, CD20 and CD56 positive cells. Subsequently, HLA-DR positive cells were identified. This subpopulation was then analysed for blood dendritic cell assay (BDCA) positivity. Initially, BDCA-1 was used exclusively. This marker is associated with myeloid or conventional dendritic cells. When very few MERTK^{+ve} cells were identified within this population, additional antibodies were added for BDCA-2, BDCA-3 and BDCA-4 in order to capture the broadest possible dendritic cell population, including those of plasmacytoid lineage (**Fig. 6.7a**).²¹²

Despite the application of additional dendritic-cell markers, very few MERTK^{+ve} events were identified in MS patients or control subjects. There was no statistically significant difference in the median fluorescent intensity, number or proportion of MERTK^{+ve} events between MS relapse and remission, nor was there a significant difference compared to control subjects at either time point (**Fig. 6.7b-d**).

a.



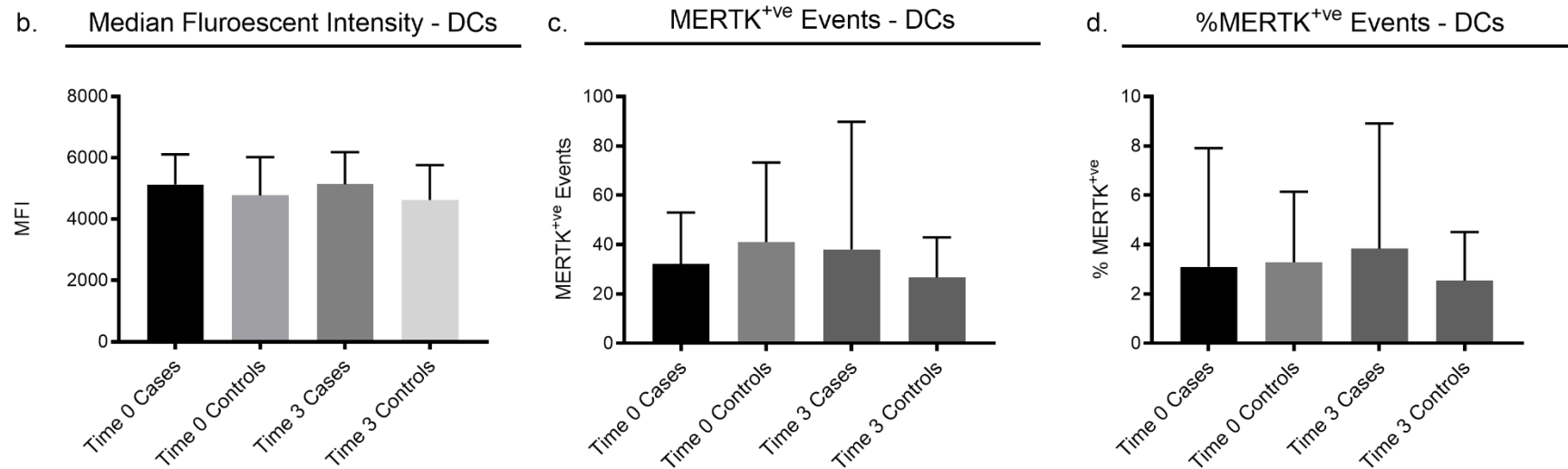
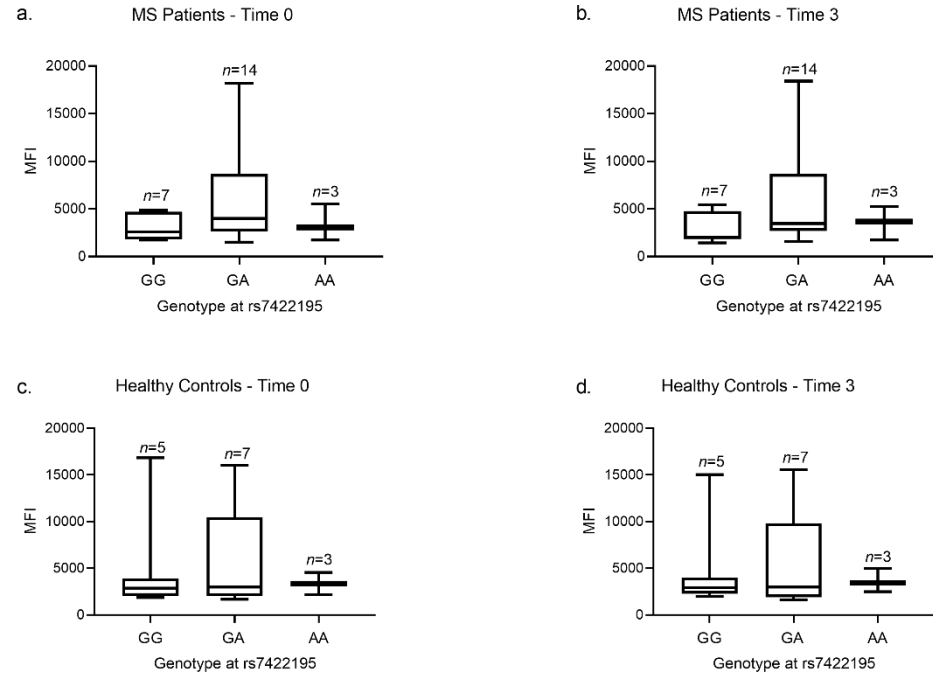


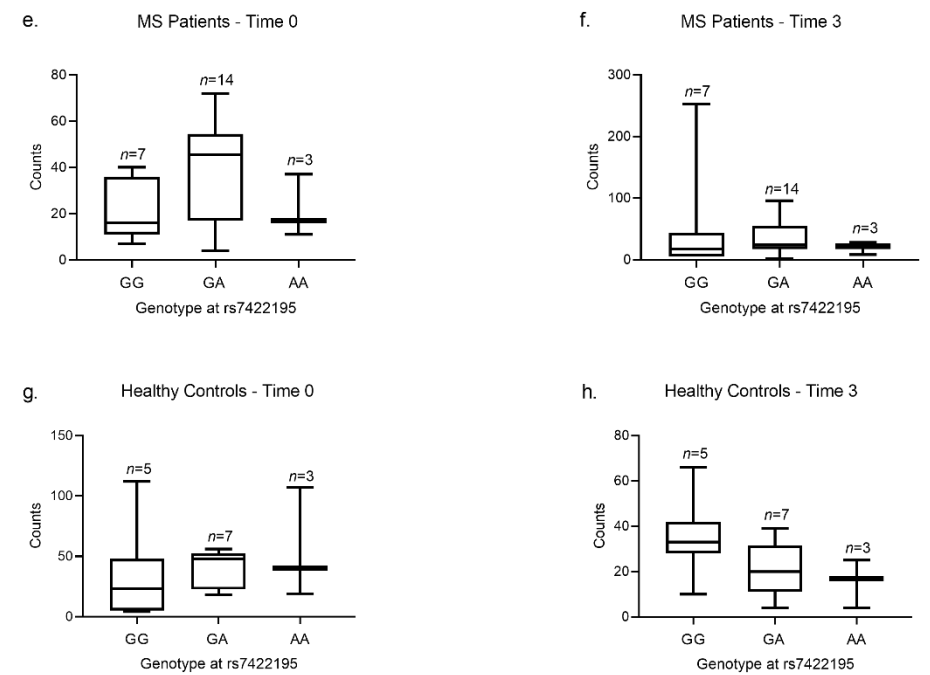
Figure 6.7. MERTK expression in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells during MS relapse or remission. **a.** Gating strategy for the identification of MERTK⁺ dendritic cells. **b.** Median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells at study entry (Time 0) and after a period of a least three months (Time 3); **c.** MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells at Time 0 and Time 3; **d.** Percentage of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells at Time 0 and Time 3.

In keeping with the analysis of MERTK⁺ monocytes, the results of the dendritic cell analysis were stratified by the MERTK polymorphism at rs7422195 in order to allow for sub-group analysis. While this analysis was limited by the very low number of events available for analysis, no statistically significant difference ($p>0.05$) in the median fluorescent intensity (**Fig. 6.8a-d**), number (**Fig. 6.8e-h**) or percentage of MERTK⁺ events (**Fig 6.8i-l**) between MS relapse and remission was identified, nor was there a significant difference compared to control subjects at either time point.

Median Fluorescent Intensity - DCs



No. of MERTK⁺ Events



% MERTK⁺ Events - DCs

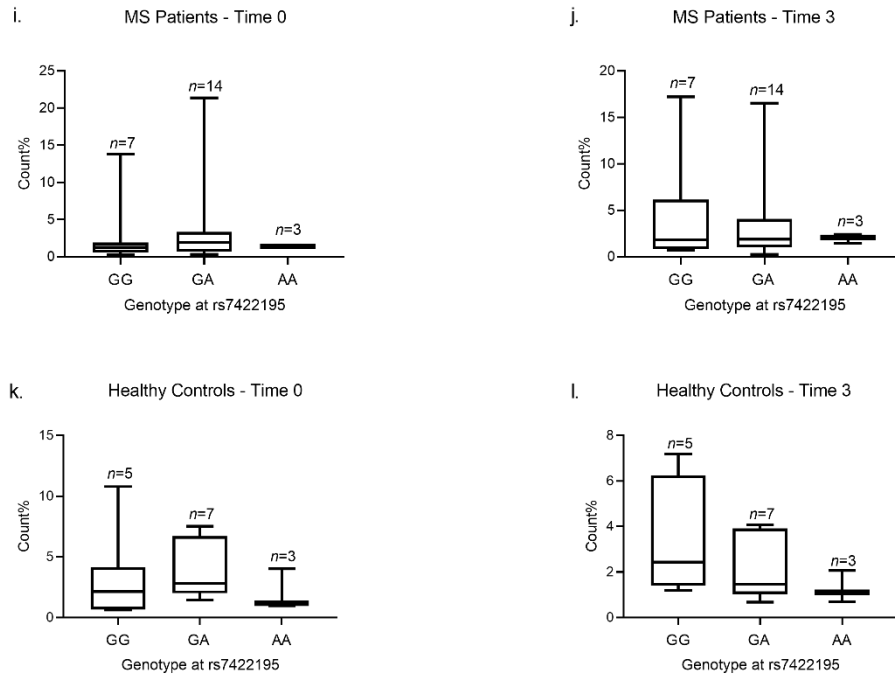


Figure 6.8. No difference in MERTK expression in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells when stratified by genotype at rs7422195 in MS relapse or remission. a. Median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0, stratified by genotype at rs7422195; b. Median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 3, stratified by genotype at rs7422195; c. Median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 0, stratified by genotype at rs7422195; d. Median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 3, stratified by genotype at rs7422195; e. MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0, stratified by genotype at rs7422195; f. MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 3, stratified by genotype at rs7422195; g. MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 0, stratified by genotype at rs7422195; h. MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 3, stratified by genotype at rs7422195; i. Percentage of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0, stratified by genotype at rs7422195; j. Percentage of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 3, stratified by genotype at rs7422195; k. Percentage of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 0, stratified by genotype at rs7422195; l. Percentage of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in healthy controls at Time 3, stratified by genotype at rs7422195. One-way ANOVA with Tukey's multiple comparison correction, $p>0.05$. Grouped expression data are plotted as Tukey box and whiskers.

An analysis of paired results on an individual basis demonstrated a high degree of consistency between median fluorescent intensity, number and proportion of MERTK^{+ve} events at both time points in MS patients (**Fig. 6.9b-d**) and control subjects (not shown).

MS Patients - Dendritic Cells

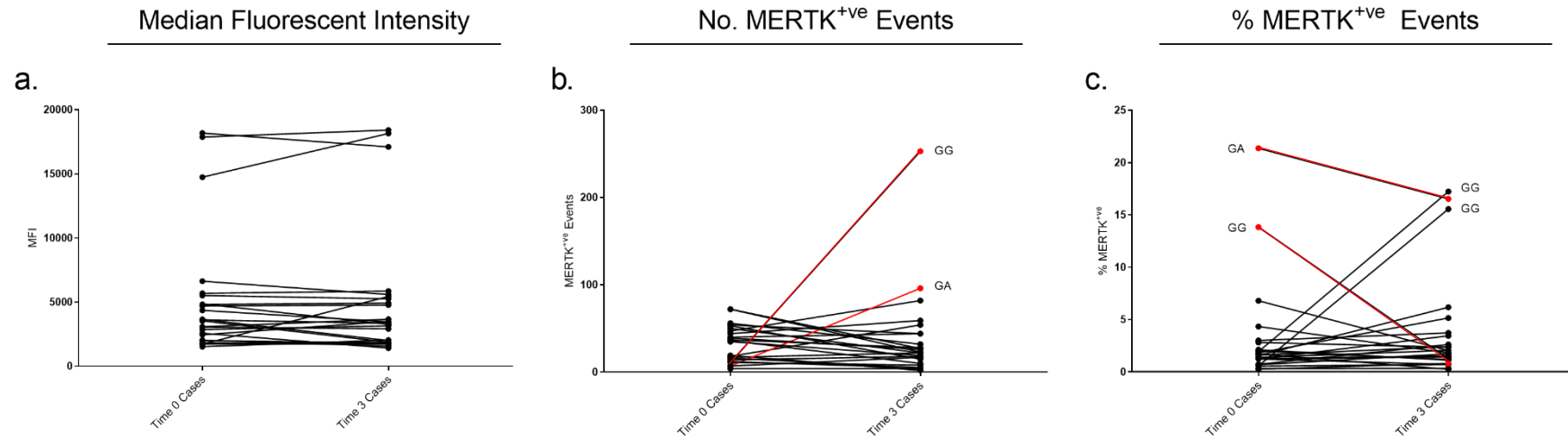


Figure 6.9. Analysis of paired dendritic cell results from each study participant did not identify relevant sub-groups based on the MERTK genotype at rs7422195. a. Paired results for median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0 and Time 3; **b.** Paired results for MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0 and Time 3; **c.** Paired results for proportion of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in MS patients at Time 0 and Time 3; **d.** Paired results for median fluorescent intensity of MERTK⁺ cells in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in control subjects at Time 0 and Time 3; **e.** Paired results for MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in control subjects at Time 0 and Time 3; **f.** Paired results for proportion of MERTK⁺ events in LIN^{-ve}HLA-DR⁺BDCA1-4⁺ dendritic cells in control subjects at Time 0 and Time 3. Genotype at rs7422195 noted for selected paired results.

6.5 Histochemical Analysis of MERTK Expression in Secondary Progressive Multiple Sclerosis

Through a collaboration with Associate Professor Ranjan Dutta at the Cleveland Clinic Lerner College of Medicine, histochemical analysis of MERTK expression in chronic active demyelinating lesions was undertaken. All autopsy specimens were from patients with secondary progressive MS. This allowed for the correlation of MERTK expression by monocytes with the degree of inflammatory infiltrate in each specimen. In addition, as the *MERTK* polymorphism at rs7422195 was known for each patient, substratification based on *MERTK* allele was also performed.

Table 6.2: Demographic Information for Cleveland Clinic Samples

<i>MS Patients (n=8)</i>	
<i>Age (Years)</i>	56.6 ± 9.3
<i>Sex (F:M)</i>	3:5
<i>Genotype at rs7422195</i>	
GG	4
GA	2
AA	2

A representative area at the border of each lesion was identified and measured (150px x 150 px, 380 μ m²). Intra-and extra-lesional counts of MERTK⁺ve and MHC-II⁺ve cells were then taken. No correlation was seen between the degree of MHC-II infiltration and the number of MERTK⁺ve cells ($R^2=0.066$, $p=0.36$)(**Fig.6.10a**). In addition, there were no genotype-specific differences in either MERTK⁺ve or MHC-II⁺ve cells. MERTK and MHC-II expression were stable across genotypes (**Fig.6.10b,c**).

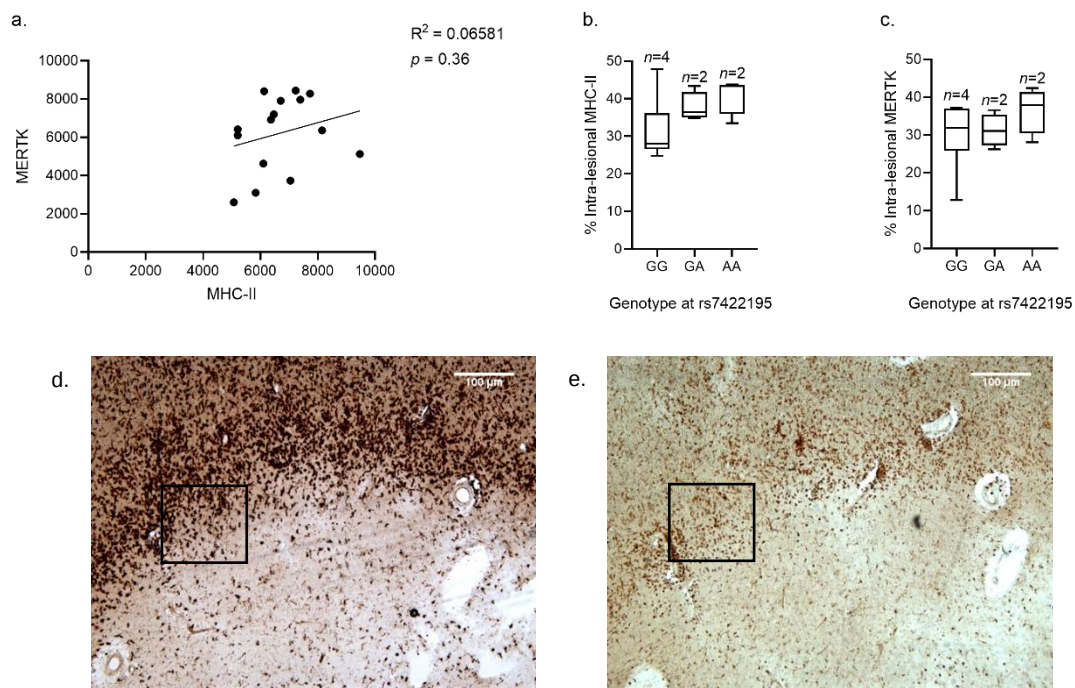


Figure 6.10. Histochemical Analysis of Chronic Active Demyelinating Lesions in Patients with Secondary Progressive Multiple Sclerosis. **a.** Simple linear regression of MERTK⁺ and MHC-II⁺ cells/mm², ($R^2=0.06581$, $p=0.36$); **b.** Proportion of intra-lesional MHC-II⁺ cells, stratified by MERTK genotype at rs7422195; **c.** Proportion of intra-lesional MERTK⁺ cells, stratified by MERTK genotype at rs7422195; **d-e.** Representative photomicrographs of MHC-II (d) and MERTK (e) staining, with representative areas of interest identified.

6.6 Discussion

The present study demonstrates that cell surface expression of MERTK by classical or non-classical monocytes, as assessed by flow cytometric analysis, does not correlate with clinical activity in MS. Specifically, there was no difference in MERTK expression during suspected relapse and after a period of at least three months when participants reported no active relapse activity. Moreover, there was no difference between MERTK expression during suspected relapse and MERTK expression by control subjects. The inflammatory activity of MS occurs discretely within the central nervous system. Assays that attempt to assess relapse activity by examination of peripheral blood specimens may not be able to capture real-time changes in MERTK expression, as peripheral monocytes may have crossed an incompetent blood-brain barrier in order to participate in inflammatory activity. A model in which MERTK expression is induced in a monocyte

population of interest may be required to reveal differences in cell activation and downstream signalling between MS patients and healthy control subjects.

Further substratification by the MERTK polymorphism rs7422195 did not identify changes in expression based on allelic representation. It is notable that MS patients carrying the minor (A) allele of rs7422195 demonstrated a trend towards lower proportions of MERTK⁺ cells compared to GG and GG counterparts. This is in distinction to published data from laboratory colleagues and, as noted above, was not accounted for by different handling techniques employed in the current study, certainly among CD14⁺ cells. Comparison among fresh and frozen CD16⁺ cells did note an increase in the proportion of MERTK⁺ cells in the frozen samples, up to two fold for the GG allele. However, given the relative paucity of CD16⁺ events, I hypothesise that this result represents stochastic variation. While the expected frequency of the minor (A) allele (3/25, 12%)-frequency of the minor allele is 0.375 in this study agrees with earlier reports, the smaller sample size available here may account for inconsistency in MERTK expression. In keeping with published data, it was also noted that non-classical monocytes expressed a greater proportion of MERTK⁺ cells compared to classical monocytes.¹⁴² These CD16⁺CD14^{LO} monocytes appear phenotypically distinct as they are not implicated in phagocytosis and the production of reactive oxygen species. Rather, they produce TNF- α , IL-1 β and CCL3 in response to viruses and immune complexes containing nucleic acids, via a pro-inflammatory pathway. Accordingly, they have been ascribed a role in local tissue surveillance,²¹³ a role which would accord with the known functions of the MERTK receptor.

Statistical analysis of dendritic cell MERTK expression was limited due to the scarcity of events on flow cytometry. However, in keeping with the monocyte analysis set out above, no difference in peripheral expression of MERTK was identified in patients experiencing MS relapse activity. In addition, there was no difference in expression between MS patients and control subjects at either time point. Prior published analyses of dendritic cell MERTK expression have largely relied on an inducible dendritic cell

model in mice. Specifically, bone-marrow derived PBMCs are exposed to granulocyte-macrophage colony stimulating factor for seven days in order to produce dendritic cells.^{132, 151, 214} While these studies report an abundance of MERTK expression by dendritic cell populations, their applicability to the present study is limited. Reports of MERTK expression by dendritic cells in humans are very limited. Published data by Hilliard and colleagues did report human dendritic cell MERTK expression but that work relied on a different flow cytometry gating strategy and did not utilise highly-specific dendritic cell markers such as BDCA.¹⁴² In addition, only MFI was reported in that work, with no assessment of the number or proportion of MERTK⁺ dendritic cells.

Accordingly, the novel finding set out in this study that absolute levels of MERTK expression by peripherally circulating dendritic cells is very low is intriguing. Demarginalised, circulating dendritic cells are likely inactive and accordingly not induced to express high levels of MERTK. Such expression may be required only in the context of antigen presentation to adaptive immune cells, where MERTK serves as a negative regulatory of dendritic cell activity.¹³² Moreover, in the setting of central inflammatory demyelination, it is likely that activated dendritic cells will not be available for peripheral sampling, as they have extravasated into the CNS through an incompetent blood-brain barrier.

Finally, histochemical analysis did not identify a correlation between inflammatory infiltrate (as characterised by MHC-II staining) and the degree of monocytic MERTK expression. Moreover, there was no difference in MERTK⁺ monocyte counts in chronic, active demyelinating lesions with reference to MERTK polymorphisms at rs7422195, despite prior data that identified variable expression patterns based on *MERTK* allele.¹⁵³ Given the chronicity of these lesions, it can be hypothesised that inflammatory processes may have reached a steady-state in which efferocytic processes have reached maturity, obscuring differences in MERTK signalling during the effector phase of lesion development.

Taken together, these data highlight challenges in employing assays that sample the periphery to interrogate CNS disease processes. While the animal data presented in preceding chapters confirm an important role for *Mertk* in response to central inflammatory demyelination, demonstrating correlation in human subjects remains challenging. The use *ex-vivo* models that permit direct manipulation of MERTK expression by human innate and adaptive immune cells may be a necessary step in further elucidating the role of this receptor in human disease.

CHAPTER 7: CONCLUSIONS

With this thesis, I aimed to interrogate the role of the receptor tyrosine kinase MERTK in the development of central nervous system inflammatory demyelination. In particular, I aimed to determine whether abrogation of MERTK expression would alter the outcomes of a frequently employed mouse-model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). In addition, I sought to determine whether changes in monocyte or dendritic cell expression of MERTK might correlate with disease activity in patients experiencing MS relapse.

This work built on foundations provided by my laboratory colleagues and doctoral supervisors who established that the *MERTK* gene is associated with MS risk and that the reduction of GAS6, the primary ligand of MERTK, alters outcomes of cuprizone-induced demyelination in mice. More broadly, MERTK and the other TAM (TYRO3, AXL, MERTK) receptors have been associated with a wide range of autoimmune conditions and their role in the maintenance of homeostasis through the removal of apoptotic cells and debris has been established.

The results in this thesis confirm the complexity of MERTK signalling. They also demonstrate the importance of assessing sexually dimorphic immune responses when animal models are deployed to investigate autoimmune disease. Importantly, the work in this thesis confirms a role for MERTK in altering the outcomes of inflammatory demyelination in a sex-specific manner.

Key findings of my research efforts include:

- Heterozygote deletion of *Mertk* in CD11⁺ cells worsens the clinical severity of EAE in male mice, demonstrating for the first time that changes in the expression of a TAM receptor can alter the outcomes of a model of multiple sclerosis.
- Homozygous deletion of *Mertk* in CD11c⁺ cells does not result in EAE exacerbation in male mice; this seemingly counter-intuitive result likely reflects

experimental factors related to genetic drift and loss of experimental phenotype, although a hormetic response to MERTK expression cannot be excluded and requires further interrogation.

- Heterozygote deletion of *Mertk* in CD11⁺ cells does not alter the outcomes of cuprizone-induced demyelination based on clinical or immunohistochemical assessment, suggesting that CNS-resident CD11c⁺ cells (a microglial subset) are not implicated in the clinical exacerbation observed in EAE cohorts.
- Sexually dimorphic responses to EAE are evident regardless of genotype. It has perhaps been under-reported that male C57BL/6 mice with and without genetic modification experience a more severe clinical response to EAE induction, based on an audit of experiences within my parent laboratory and from a review of published literature. This finding has important implications for the use of this animal model in the development of human therapeutics.
- Cellular differences identified in the spinal cords of male and female mice following EAE induction may explain these clinical differences, with female mice demonstrating elevated numbers of IBA1⁺ microglia and oligodendrocytes, suggesting an enhanced reparative phenotype.
- In human subjects, MERTK expression by monocytes and dendritic cells does not correlate with periods of increased inflammatory activity in MS. MERTK expression by circulating dendritic cells is very limited.

The development of the *Mertk*^{fl/wt} and *Mertk*^{fl/fl} mice was stimulated by human genetics data implicating the *MERTK* gene in MS susceptibility and severity. While experimental data demonstrating changes in outcomes following GAS6 deletion in both EAE and cuprizone models of demyelination were encouraging, these experiments were not able to interrogate the specific mechanism driving altered phenotypic and cellular outcomes, as the GAS6 ligand can signal through all three TAM receptors. Accordingly, a MERTK-specific approach was adopted and the data presented here confirm a role for this receptor tyrosine kinase in the propagation of immune responses following EAE induction in male mice. This novel finding is encouraging and further experimentation

to confirm the central role for MERTK in modulating immune responses in EAE is warranted. Further investigation is clearly needed to determine if dendritic cells or microglia are the relevant effector cells responsible for the phenotype seen in early cohorts following EAE induction. An intriguing potential model involves the use of dendritic cell transfer experiments to assess the anti-inflammatory capacity of *Mertk* deficient dendritic cells to ameliorate the clinical effects of EAE. In WT mice, repeated immunisation with MOG antigen produces a population of tolerogenic CD11b⁺CD11c⁺ dendritic cells that can dramatically improve outcomes following transfer to animals at EAE disease peak. Compromise of that tolerogenic capacity in *Mertk* deficient dendritic cells would confirm a role for that cell population in the clinical phenotype identified with these data.

The data presented in this thesis also demonstrate the inherent complexities of TAM receptor signalling. At the outset of these experiments, I hypothesised that TAM signalling by the immune system would result in the down-regulation of pro-inflammatory responses and that abrogation of TAM expression would result in more severe manifestations of auto-immune phenotypes. These expectations stem from early experiments with TAM triple knock-out animals, where autoimmune phenotypes were rapidly manifest and are supported by multiple experiments with discrete receptor deletion. Yet the experimental data presented here demonstrate a more complex reality. Responses to TAM silencing or ligand deprivation depended on sex and immune context. While the more severe clinical manifestations of EAE in male mice may have modulated over time, the absence of clinical signal in female mice was very consistent, demonstrating striking sexual dimorphism. Furthermore, as the resting and EAE-challenged immune cell proportions are subtly different between sexes, the immune context in which EAE is induced may also be significant. Clearly, our understanding of the way in which MERTK expression and GAS6 signalling modulate inflammatory demyelination is incomplete. While it was outside the scope of this work to elucidate the specific mechanisms of sexually dimorphic outcomes in EAE, repeat experiments in

oophorectomized and sham surgery female cohorts would test the hypothesis that sex hormones are key to the variation in clinical outcomes.

While clinical outcomes varied in a sex-specific manner, similar differences were not detected by examination of cellular response through immunohistochemical analysis. In particular, there were no differences in the degree of inflammatory infiltrate in the lumbar spinal cord of EAE-treated mice with reference to genotype or sex when analysed at disease peak. The finding of a striking difference in CD3⁺ and CD11c⁺ cell counts in male CD11c-Cre^{-/-}/*Mertk*^{fl/fl} when EAE was terminated during the effector stage of the experiment (12 dpi) is intriguing and suggests there might be substantially increased immune infiltration in these animals compared to *Mertk*-deficient littermates. This cellular infiltrate may represent a regulatory (T_{reg}), anti-inflammatory response to inflammatory demyelination but the limited number of animals in these cohorts mandates further investigation with repeat experimentation.

While encouraging progress was made with regard to our understanding of TAM receptor signalling in experimental demyelination, results from human subjects were more circumspect. While *MERTK* may be a risk gene for MS susceptibility, its expression at the protein level on circulating monocytes and dendritic cells does not seem to correlate with periods of enhanced inflammatory activity in MS. The novel finding set out in this study that absolute levels of MERTK expression by peripherally circulating dendritic cells is very low is intriguing. Demarginalised, circulating dendritic cells are likely inactive and accordingly not induced to express high levels of MERTK. Such expression may be required only in the context of antigen presentation to adaptive immune cells, where MERTK serves as a negative regulatory of dendritic cell activity.

The human data presented here demonstrate the challenges in employing assays that sample the periphery to interrogate CNS disease processes – it is likely that the activated monocytes and dendritic cells that we hoped to analyse were not be available for peripheral sampling, as they had already extravasated into the CNS through an

incompetent blood-brain barrier in the setting of increased MS disease activity. Analysis at the RNA level and via an *ex-vivo* *MERTK*-inducible model may be required in order to more fully investigate the role of this receptor in the development and perpetuation of autoimmune inflammatory demyelination. With these experiments, I have also developed a valuable biobank of human monocytes and dendritic cells collected during MS relapse and remission with matched healthy controls that can be used for further experimentation.

Taken together, the data in this thesis offer compelling evidence that *MERTK* is an important regulator of the outcomes of inflammatory demyelination. They justify further research efforts in this area to better understand the mechanism of these disease states and to develop therapeutic targets for the treatment of MS. The sexually dimorphic response to murine EAE, moreover, offers important echoes with the human condition and further interrogation of the aetiology of this dimorphism may assist in understanding sex-specific differences in autoimmune disease more broadly.

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