The effects of fumarate treatment on myeloid cells in multiple sclerosis

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TABLE OF CONTENTS PAGE

PART 1- INTRODUCTION

Acknowledgements vi

Contributions viii

Abstract ix

Resumé x

Terminology of Note/ Terminologie importante xi

Introduction xii

PART 2- LITERATURE REVIEW *Michell-Robinson et al. 2015* 1

Multiple Sclerosis: Overview 3

The Peripheral Myeloid Contribution to Central Nervous System Inflammation in Multiple Sclerosis 5

Unique Aspects of Central and Peripheral Myeloid Cell Populations 8

Figure 1: Relative responses of microglia, monocytes, and monocyte-derived (*in vitro*-differentiated) macrophages to 100 ng/mL lipopolysaccharide for six hours, *in vitro.* 10

Microglia:

In the Developing Central Nervous System 11

Pathology and Homeostasis in the Adult Central Nervous System 15

Neurobiology and Endogenous Repair in the Adult Central Nervous System 18

Microglia in Multiple Sclerosis: Summary 21

Fumarate Treatment for Multiple Sclerosis 23

Figure 2: Models of Dimethyl and Monomethyl Fumarate Structure 24

PART 3- THE EFFECTS OF FUMARATE TREATMENT ON MYELOID CELLS IN MULTIPLE SCLEROSIS *Michell-Robinson et al., 2015* **26**

Introduction **28**

Methodology **30**

Results **34**

Figure 3: Anti-inflammatory and anti-oxidative effects of DMF treatment in monocytes isolated from RRMS patients **35**

Figure 4: DMF but not MMF treatment *in vitro* reduces LPS-induced cytokine production and anti-oxidant gene expression in monocytes **37-38**

Figure 5: DMF *in vitro* is cytotoxic to monocytes from healthy controls and patients **39**

Figure 6: DMF treatment *inhibits* LPS-induced IL-6 and *induces* HMOX-1 expression in monocyte-derived macrophages (MDMs) in absence of cytotoxicity **40-41**

Figure 7: Neither DMF nor MMF is toxic to monocyte-derived macrophages or microglia *in vitro* **42-43**

Figure 8: DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression and *induces* HMOX1 expression in human fetal microglia **44-45**

Figure 9: DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression, and *induces* HMOX1 expression in human adult microglia **47-48**

Figure 10: HCAR2 expression in human fetal microglia and astrocytes **49**

Discussion **50**

Conclusion **53**

PART 4- APPENDICES

Appendix 1: Cell Culture Methodology **55**

Appendix 1A: Preparation of Pure Cultures of Human Microglia for *In Vitro* Experimentation **52**

Appendix 1B: Isolation of Monocytes from Peripheral Blood Mononuclear Cells by Density Gradient Centrifugation and *in vitro* differentiation of Monocyte-Derived Macrophages **60**

Appendix 1C: Preparation of Pure Cultures of Human Fetal Astrocytes for *In Vitro* Experimentation **62**

Appendix 2: Assay Methodology

Appendix 2A: Methods for Quantification of RNA by Real-Time, Quantitative Polymerase Chain Reaction **63**

Appendix 2B: Colorimetric Enzyme Linked Immunosorbance Assay (ELISA) **67**

Appendix 2C: Methods for Staining and Quantification of Cellular Protein by Flow Cytometry **69**

Appendix 2D: Methods for Immunofluorescence Staining and Imaging **72**

BIBLIOGRAPHY **74**

PART 1: INTRODUCTION

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Abstract

Our aim was to analyze the impact of fumarates on the anti-inflammatory and anti-oxidant profiles of human myeloid cells including monocytes, blood derived macrophages, and brain derived microglia *ex vivo* and/or *in vitro*. We demonstrate that monocytes from multiple sclerosis (MS) patients receiving Dimethyl fumarate (DMF) therapy, as well as patients receiving Fingolimod (FTY720) therapy, had reduced relapsing-remitting multiple sclerosis (RRMS)- associated inflammatory and anti-oxidant gene expression as assessed by miR-155 (inflammatory), HMOX1 and OSGIN1 (anti-oxidant) expression, respectively. Anti-oxidant NQO1 expression was not significantly affected in patients undergoing therapy. The observed changes occurred without affecting monocyte number or viability. *In vitro studies* made use of DMF and *in vivo*-produced DMF metabolite Monomethyl fumarate (MMF). *In vitro*, DMF in the 10-50 μM range induced cytotoxicity of monocytes but not of macrophages or microglia. In macrophages and microglia, DMF inhibited the inflammatory response to lipopolysaccharide (LPS) and increased expression of the anti-oxidant gene HMOX1 without upregulation of NQO1 or OSGIN1. MMF had no measurable anti-inflammatory or anti-oxidant effect on myeloid cells although its specific receptor HCAR1 was present on microglia. Our observation that both DMF and FTY720 therapy reduce systemic myeloid cell inflammatory and anti-oxidant gene expression *in vivo* suggests that this may be an indirect effect reflecting overall reduction in disease activity. Our *in vitro* studies suggest that any direct effects of fumarate therapy on myeloid cells are mediated via DMF. Our finding that HMOX1 is specifically upregulated by DMF in microglia, along with the lack of upregulation in monocytes undergoing cytotoxic responses to DMF, indicates that HMOX1 upregulation is a direct rather than a stress-induced response. These results indicate a greater *in vitro* effect of DMF versus MMF on all primary human myeloid cells although there is a significant difference in sensitivity of different myeloid populations to these agents. This study raises the question of which mechanism is responsible for the reduced disease activity in patients receiving this therapy.

Resumé

Notre but est d'analyser l'impact des fumarates sur les réponses anti-inflammatoires et anti-oxydantes des cellules myéloïdes humaines incluant les monocytes, macrophages et microglies *ex vivo* et *in vitro*. Nous démontrons que les monocytes provenant de patients atteints de la sclérose en plaques (SP) traités au dimethyl fumarate (DMF), ou au FTY720, démontrent une réduction de l'expression de miR-155 associée à l'inflammation, et à la réponse anti-oxydante associée aux gènes HMOX1 et OSGIN1, sans pour autant altérer le nombre ni la viabilité des monocytes. Nos études *in vitro* démontrent que le DMF (a concentration variant entre 10 et 50 µM) a induit un effet cytotoxique dans les monocytes, mais pas dans les macrophages, ni les microglies. Le DMF inhibe la production de cytokines en réponse au LPS et augmente l'expression de HMOX1 sans augmentation coïncidente de NQO1 ni d'OSGIN1. MMF n'a pas eu d'effet mesurable dans ce contexte expérimental, malgré la détection du récepteur HCAR1 à la surface des microglies. L'observation relatant la réduction des gènes pro-inflammatoires (miR-155) et anti-oxydantes (HMOX1, OSGIN1) *in vivo* en réponse aux thérapies DMF et FTY720, indique que l'effet observé reflète une diminution de la phase aigue de la maladie. Les observations générées *in vitro* suggèrent que tout effet pouvant être médié par la thérapie au fumarate sur les cellules myéloïdes sont induits par le DMF. Nos résultats suggérant l'augmentation spécifique de HMOX1 par le DMF dans les microglies. Cela indique que la régulation à la hausse de HMOX1 est un effet direct en réponse au DMF, et non dû au stress. Nos résultats montrent un effet plus important *in vitro* de DMF comparé au MMF, en particulier dans les cellules myéloïdes primaires humaines. Par contre, nous reportons une différence significative quant à la susceptibilité des différentes sous populations myéloïdes aux agents thérapeutiques fumarates. Les mécanismes d'action responsables de la diminution de la phase active de patients traités au DMF restent à mystère à élucider.

Terminology of Note/ Terminologie importante

MS - Multiple Sclerosis

RRMS – Relapsing-Remitting Multiple Sclerosis

LPS - Lipoloysaccharide

DMF – Dimethyl Fumarate

MMF – Monomethyl Fumarate

FTY720 – Fingolimod

miR-155 – MicroRNA 155

HMOX1 – Heme Oxygenase 1

NQO1 – NAD(P)H dehydrogenase, quinone 1

OSGIN1 – Oxidative Stress Induced Gene 1

Introduction

The following work is an evaluation of the effects of currently approved fumarate treatment on the myeloid cell subsets associated with multiple sclerosis pathology. We have observed the effects of this treatment both *ex vivo,* and *in vitro* to evaluate current pharmacodynamic hypotheses. Specifically, that fumarate treatment induces anti-oxidant and anti-inflammatory responses in key cell subsets in MS. To this end, we have measured the expression of markers of inflammation and anti-oxidant activity in MS patients both on and off therapy, as well as healthy controls. Additionally, we included FTY720 patients as a control for the indirect effects of lowering overall disease activity which could be reflected in the inflammatory and oxidative status of patient monocytes. In such a case (as was observed), two therapies with very different pharmacodynamics would induce the same effects on markers of inflammation and oxidative stress. Furthermore, we have evaluated the inflammatory function of human myeloid cells including monocytes, macrophages, and microglia, in the presence of both major metabolic species of fumarate treatment, in an effort to better understand the separate pharmacodynamics of these drug species in specific myeloid cell subsets. To this end, we have chosen both sub-physiological (10 μM) and superphysiological (50 μM) doses of fumarates for use *in vitro*. An in depth introduction to the research is presented on page 28.

The thesis is organized into three parts following the introduction. Part 2 is a literature review examining the role of both peripheral and central myeloid cells in multiple sclerosis, as well as an overview of multiple sclerosis disease activity. Also included are review sections from *Michell-Robinson et al., 2015,* on microglial physiology both as an adult and in fetal development. These sections serve to deepen the reader's understanding of microglial physiology as homeostatic regulators of the neural environment, and hopefully to understand better the relationship between microglial dysregulation and pathology in the brain at different stages of life. Part 3 is the research manuscript introduced above: *The effects of fumarate treatment on myeloid cells in multiple sclerosis*, which was in final review at *Annals of Clinical and Translational*

Neurology at the time of thesis submission. Finally, Part 4 contains appendices and comprehensive references for further, in-depth review of the presented topics.

PART 2: LITERATURE REVIEW

The following literature review has been adapted from the publication cited below in Brain[1]. Unpublished information on the topic of non-microglial myeloid cells and multiple sclerosis pathology has been added where necessary, and the text has been adapted for thesis formatting and style.

Roles of Microglia in brain development, tissue maintenance and repair

***Brain*, 2015**

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Abstract:

The emerging roles of microglia are currently being investigated in the healthy and diseased brain with a growing interest in their diverse functions. In recent years, it has been demonstrated that microglia are not only immunocentric, but also neurobiological and can impact neural development and the maintenance of neuronal cell function in both healthy and pathological contexts. In the disease context, there is widespread consensus that microglia are dynamic cells with a potential to contribute to both central nervous system damage and repair. Indeed, a number of studies have found that microenvironmental conditions can selectively modify unique microglia phenotypes and functions. One novel mechanism that has garnered interest involves the regulation of microglial function by microRNAs, which has therapeutic implications such as enhancing microglia-mediated suppression of brain injury and promoting repair following inflammatory injury. Furthermore, recently published articles have identified molecular signatures of myeloid cells, suggesting that microglia are a distinct cell population compared to other cells of myeloid lineage that access the central nervous system under pathological conditions. Thus, new opportunities exist to help distinguish microglia in the brain and permit the study of their unique functions in health and disease.

Multiple Sclerosis: Overview

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory disease of the central nervous system. In Canada, MS has an estimated prevalence of 240 in 100,000 individuals. It is the primary neurological cause of disability in the developed world [2-4]. MS is associated with both genetic and environmental risk factors [5]. It is generally accepted that more than one genetic risk alleles interacts with environmental factors resulting in disease, however, our understanding of the specific mechanisms of MS pathology is incomplete. MS patients follow one of several general clinical patterns separated broadly into relapsing and progressive disease courses. The large majority of patients suffer from a relapsing-remitting disease course which is followed by transition to a progressive form of MS in the latter part of the illness. The relapsing-remitting disease course is characterized by neurological episodes thought to be driven by cellular activity within central nervous system (CNS) lesions- the primary site of immune attacks on myelinating cells (oligodendrocytes) and neurons [6]. Knowledge of the cellular mechanisms underlying the innate, inflammatory immune response and adaptive immune responses in the context of relapsing-remitting MS disease activity has led to the generation of multiple therapeutic compounds [7]. Current therapeutic aims include the prevention or reduction of relapses and delaying the onset of disability through the modulation of peripheral immune cells, reduction of their entry to the CNS, and-- in a minority of cases-- direct modulation of CNS cell properties [8]. The heterogeneity of disease course and patient response to MS therapeutics (both adverse and positive), combined with the need for better understanding mechanisms of CNS damage in MS, has influenced the continual study of approved and potential MS therapeutics. To this end, the use of human peripheral and CNS tissue has proved itself a rare but indispensible resource to the MS research community.

Despite the heterogeneous clinical course observed between MS patients, all MS patients are known to have focal demyelinated plaques with partial axonal preservation, reactive glial scar formation, and diffuse damage throughout the so-called “normal-appearing” white and grey matter (NAWM and NAGM). The interaction and relative contribution of these changes in the context of global pathology varies considerably

between clinical courses and patients. How immune cells access the CNS and mediate the observed damage to neural tissues has been extensively studied, though the trigger of autoimmune attacks in MS remains unknown [9-28]. It is thought that the trigger of MS may be one or more infectious or other environmental stimuli. Of the risk alleles identified in MS genome-wide association studies, most produce molecules associated with immune function, however, a number are specifically involved in nervous system functionality [29, 30]. The anatomy of the human MS lesion has been well described; it is known to contain immune cells from the peripheral blood (macrophages, T cells, B cells, etc), activated microglia, and reactive astrocytes which influence demyelination, remyelination processes, and tissue damage [6, 31]. Therefore, the study of MS necessarily involves studying the entrance of immune cells into the CNS and their interactions with neural cell types including glial cells. In this respect, myeloid cells are particularly interesting as they are both CNS-resident (microglia) and peripherally-derived (monocytes, macrophages), with implications in MS pathology during all stages of the disease.

The Peripheral Myeloid Contribution to Central Nervous System Inflammation in Multiple Sclerosis

Different subsets of myeloid cells can be found in all major tissues of the CNS. Microglia are found within the CNS parenchyma and are a self-maintained population of myeloid cells under steady-state conditions; they will be discussed in detail in further sections. Macrophages can be found at the interfaces of the CNS with peripheral tissues: in the choroid plexus, perivascular space, and in the meninges. These populations of macrophages are maintained throughout life by entry and differentiation of circulating monocytes, and are hence termed monocyte- or peripheral-derived macrophages. In the inflamed CNS, monocyte-derived macrophages are known to infiltrate and mediate effector functions which have both beneficial and detrimental effects on the parenchyma [32].

Monocytes are circulating innate immune cells which evolve from stem cell precursors found in the bone marrow. Once they have emigrated into the blood, monocytes have at least two distinct phenotypes. The human population of $CCR2^{lo}CD14^{hi}CD16^{hi}$ monocytes are analogous to a population of mouse monocytes known to have a patrolling function involving a search for damage and infection along endothelial surfaces [33, 34]. In contrast, a second population of $CCR2^{hi}CD14^{hi}CD16^{lo}$ human monocytes is analogous to a mouse monocyte population that is specialized to respond to tissue inflammation [33, 34]. It is unclear whether these two subtypes of monocytes develop independently from a common progenitor or whether $CCR2^{hi}$ monocytes eventually become $CCR2^{lo}$. Monocytes are a heterogenous cell population owing to the influence of environmental factors and temporal changes associated with differentiation and maturation *in vivo*.

There are several lines of evidence indicating a crucial role for monocytes and macrophages in MS. Monocyte-derived macrophages are a key cellular component of active demyelinating lesions and are often found near transected axons[35]. Active MS lesions are characterized by the presence of myelin component digestion in myeloid cells. It is unknown whether phagocytosis is primarily accomplished by microglia or

macrophages, however, it is most likely to be both cell types as they are both capable of myelin phagocytosis *in vitro* [36-38]. Furthermore, both monocyte-derived macrophages and microglia display an activated phenotype in MS, characterized by secretion of inflammatory and toxic mediators, as well as elevated expression of co-stimulatory molecules used for antigen presentation to T cells [36, 39]. Early studies using experimental autoimmune encephalomyelitis (EAE- an animal model of MS) indicated a causal relationship between myeloid cell infiltration and disease severity. More recent studies have shown mobilization of the Ly6C^{hi} population of mouse monocytes (analogous to CCR2^{hi} discussed previously) prior to disease exacerbation and that these are crucial to disease evolution [40-43]. However, studies have not conclusively shown that this population of cells is entirely destructive. For instance, infiltration of this population of monocytes occurs coincidentally with CNS repair after lysolecithin-induced demyelination [44, 45]. Additionally, in spinal cord injury models, recruited monocytes/macrophages are known to play key roles in promoting recovery [46]. This may occur through phagocytosis of debris and secretion of trophic factors or cytokines such as interleukin 10 (IL-10). Currently, it is thought that monocyte-derived macrophages may contribute to either repair or damage of CNS tissue by assuming certain phenotypes along a spectrum characterized by the M1/M2 paradigm.

The M1/M2 experimental paradigm refers to a nomenclature which developed out of attempts to characterize the influence of environmental factors such as cytokines and chemokines on the expression of effector molecules in macrophages. It was initially discovered that macrophages have at least two major phenotypes [1, 47-49]. The M1 phenotype associates with expression of inflammatory molecules and can be induced by IFNγ and LPS, whereas an M2 phenotype associates with expression of anti-inflammatory molecules and is induced by IL-4. To date, there are several different so-called “polarization states” of macrophages and microglia which are now named by the cytokines used to induce them [1]. These have various expression profiles with implications for function, and it is assumed that the presence of these cytokines in tissues would lead to particular cellular behaviours in the context of disease. Some of these have been evaluated using animal models such as EAE, where it has been demonstrated that so-called M2 macrophages can improve disease activity [50]. Although

it is uncertain whether defined M1/M2 subtypes are present *in vivo* to the same degree that we can polarize them *in vitro* [51], striking results have been achieved using *ex vivo* polarized macrophages to ameliorate the course of EAE, while causing diverse effects in other disease models [50, 52].

Unique Aspects of Central and Peripheral Myeloid Cell Populations

Unique aspects of microglia ontogeny

Microglia are mesodermally-derived mononuclear cells of the central nervous system that originate during *primitive haematopoiesis* in blood islands within the yolk sac [34, 53, 54]. In histological sections, human microglia can be identified in the extracerebral mesenchyme as early as 4.5 gestational weeks (GW) and invade the parenchyma at approximately 5 GW [55-57]. In rodents, microglia are initially detectable as primitive tyrosine protein kinase c-kit (c-kit) positive erythromyeloid precursors at embryonic day 8 (E8) [58], consistent with prior observations that progenitors arise before E8 in rodent [53, 59]. Primitive haematopoiesis may also contribute precursors to the adult haematopoietic stem cell (HSC) population; however, *definitive haematopoiesis* which occurs within embryonic tissue is responsible for the majority of HSC development [34, 60]. HSC progeny are responsible for the circulating or 'peripheral' compartment of monocytes which infiltrate tissue during injury/immune challenge and replenish other types of tissue resident macrophages throughout life (see *Maintenance of microglia populations, microgliosis, and parenchymal precursors*). For further review, see [33, 61].

Although the functional significance of their unique ontogeny remains to be elucidated, initial insights have revealed that some differences in microglial developmental genetic programming exist as compared with peripheral cell types. Studies using proto-oncogene c-myb$^{-/-}$ (c-myb$^{-/-}$) mice, a regulatory transcription factor of hematopoiesis, demonstrate that unlike HSCs, erythromyeloid progenitors resulting from primitive haematopoiesis do not require c-*myb* for self-renewal and proliferation [62, 63]. However, interpreting these findings is complicated by the fact that c-myb regulates the colony stimulating factor 1 receptor (CSF1R), which is involved in the maintenance and differentiation of both monocytes and microglia [33]. Microglial development is further subjected to regulation by transcription factor PU.1 (PU.1) and interferon regulatory factor 8 (IRF-8), both implicated in peripheral myeloid development. CX3CR1 is commonly used for microglia lineage tracing in rodent models and is also expressed in humans [53, 63]. Kierdorf *et al*. described markers of rodent microglia prior to fractalkine receptor (CX3CR1) expression [58]. An early protein tyrosine phosphatase receptor type

C (CD45) positive, CX_3CR1 negative ($CD45^+/CX_3CR1^-$) myeloid precursor population is dependent on PU.1, while loss of IRF8 causes a maturational defect and significant apoptosis of microglia precursors before acquiring fractalkine receptor expression. IRF-8 is linked downstream of PU.1, implicating these factors in an early microglia differentiation and development cascade [58]. However, the C57Bl/6 genetic background may require PU.1 for yolk sac macrophage development, but outbred $PU.1^{-/-}$ mice do not [33, 64]. Furthermore, Minton *et al*. reported an increase in microglia number using the same IRF-8 knockouts crossed with CSF1R-eGFP C57Bl/6 mice [65, 66]. These discrepancies highlight the influence of genetic background in studies focusing on microglial ontogeny; it is unclear how these findings translate to humans. Quantitative genetic and proteomic analysis revealed that a transforming growth factor beta (TGF-β)-dependent unique microglial molecular and functional signature is present in both rodents and humans [67]. Thus, human microglia display crucial developmental differences compared to peripheral myeloid cells; for further review we suggest [68].

Similarities of microglia and peripheral myeloid cells *in vitro* and *in vivo*

When compared with peripheral myeloid cells, microglia share many phenotypic similarities. Indeed, it has been difficult to find markers to differentiate microglia from macrophages of peripheral origin in inflammatory and other conditions within parenchymal tissues. *In vitro*, much work has demonstrated that microglia respond similarly to macrophages in the context of M1/M2 paradigm cytokines, while displaying some unique differences in inducible molecular phenotype [36-38, 69]. In the human system, it is commonly observed that microglia and macrophages respond similarly to standard inflammatory inducers such as LPS, by secretion of cytokines such as Tumor Necrosis Factor (TNF), interleukin-6 (IL-6) and IL-10. Microglia are known for an IL-6 dominant response to LPS which is associated with maturation (increased in adult microglia *in vitro*), while *in vitro* differentiated macrophages respond with particularly robust production of TNF **(Fig. 1)**.

This is echoed by the previously mentioned discovery of a TGFβ-dependent molecular phenotype for microglia in the brain [67]. Among the molecules putatively named unique molecular markers for microglia is P2Y12 [38], a G-protein coupled receptor which

recognizes ADP as a ligand, that has been used to discriminate between central and peripherally derived microglia and macrophages, respectively. It is still unknown if this marker will be effective in all pathological circumstances. As a result of the relative novelty of this unique molecular signature, little is known at the current time about differential contributions of central and peripheral cell types in ongoing MS pathogenesis and evolution of the disease.

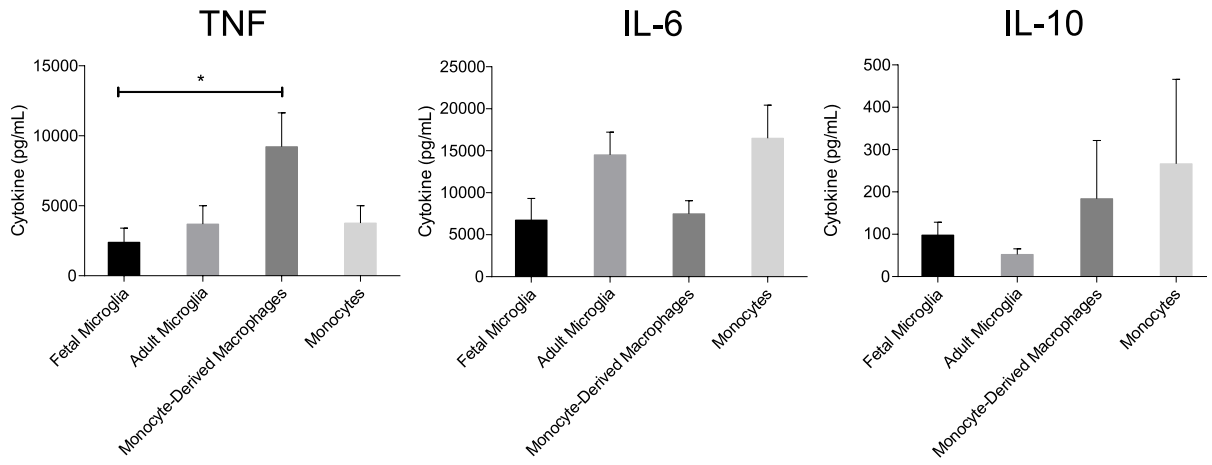

Figure 1: Relative responses of microglia, monocytes, and monocyte-derived (*in vitro*-differentiated) macrophages to 100 ng/mL lipopolysaccharide for six hours, *in vitro*. At left, TNF responses are particularly robust in monocyte-derived macrophages (MDMs) compared to microglia, while IL-6 (at middle) responses are more robust in microglia. Monocytes, macrophages and microglia all respond similarly with low levels of IL-10 induction (at left) in response to LPS stimulation. One-way ANOVA compares means of all groups against each other, $\alpha = 0.05$; * $p<0.05$, $n \geq 4$.

Microglia in the Developing Central Nervous System

The distribution of microglia in human embryonic histological sections suggests they enter the brain anlagen through the developing meninges, ventricular zone, and choroid plexus [55-57]. Initially, microglia display an amoeboid morphology and subsequently acquire a mature, ramified morphology. The developmental amoeboid morphology suggests that they are activated during development [70, 71]. Indeed, developmental microglia express chemokine receptors, and may express immunologically active cytokines, however these have other, neurobiological roles in the nervous system as discussed below [57, 58, 72, 73]. Members of the matrix metalloproteinase (MMP) family, such as MMP8 and MMP9, regulate overall microglia number and distribution in the embryonic mouse brain [58]. Indeed, immature rodent microglia upregulate certain chemokines and their cognate receptors (*e.g.* CX3CR1, CCR2, CCR1, CXCR3) upon maturation in the brain parenchyma. However, their morphology and numbers remain constant in the respective knockout animals and suggests these molecules are not necessary for distribution in development [58]. With increasing interest in differences between microglia from different brain regions, factors that regulate the migration and positioning of microglia in the developing brain will become increasingly interesting in the context of developmental disease [74-79].

Bidirectional interaction of microglia and the developing neural architecture

The human cerebral cortex is made up of 20-25 billion neurons arising from the ventricular and subventricular zone (SVZ) during embryonic development. Microglia comprise 6-18% of neocortical cells in the human brain [80, 81]. Together, the early establishment of microglia in the developing brain [53, 55-57], an activated morphological and molecular phenotype [71, 72, 82, 83], their overall prevalence [80, 81], and the finding of severe microgliosis in some neurodevelopmental disorders [71, 82, 83], all suggest the possibility of microglia-neuron crosstalk in development. Indeed, there are a number of studies finding implications for microglia in developmental contexts ranging from Autism-Spectrum Disorders [84], Obsessive Compulsive Disorder [85, 86], Schizophrenia [87,

[88], Tourette Syndrome [89], Cerebral Palsy [90], Fetal Alcohol Spectrum Disorders [91], Fragile X Syndrome [92, 93], to others still.

Soluble factors are the most apparent candidates for developmental microglia-neuron crosstalk. Inhibition of microglial activation with minocycline reduces both neurogenesis and oligodendrogenesis [72]. Neural precursors are responsive to combinations of cytokines interleukin 1 beta (IL-1β) and interferon gamma (IFNγ) released by microglia [72]. Microglia can also augment oligodendrogenesis through a combination of IL-1β and interleukin 6 (IL-6), but the effects are targeted to later stages of oligodendroglial development [72]. SVZ neural stem cell cultures from P8 mice maintain the ability to form neurospheres, but progressively lose the ability to generate new neuroblasts. Co-culture of SVZ neural stem cells from P8 mice with microglia or microglia-conditioned media rescues their ability to generate neuroblasts from these neurospheres [94]. Some have suggested that microglia from neurogenic regions activate and behave differently than those from non-neurogenic areas [73, 95]. For instance, Mosher et al. found that NPC secretory products can modulate the cytokine profile of microglia, while augmenting their phagocytosis and migration functions *in vitro*. These effects were apparently due to vascular endothelial growth factor derived from NPCs [96]. Together, these studies highlight the bidirectional nature of the microglia-neuron crosstalk in development.

Microglia may also physically regulate the number of NPCs in the developing cerebral cortex through phagocytosis. In rodents and non-human primates this occurs via preferential removal of viable neurons-- independently of apoptotic markers-- within proliferative zones and in the latter stages of cortical neurogenesis [97]. Large numbers of activated microglia are found within neural proliferative zones, while few microglia are present within the cortical plate of these animals, consistent with histological studies in humans [55-57]. Interestingly, maternal LPS administration negatively impacts the NPC population, presumably by promoting a pro-inflammatory microglia phenotype (M1). Conversely, doxycycline skews the phenotypic ratio towards an anti-inflammatory phenotype and significantly increases the NPC population in rodents [97]. The mechanisms responsible for the phagocytosis of viable neurons in the developing human CNS are currently unknown; however, relevant phagocytosis-mediators in the

adult CNS may provide initial insights (see *Neuronal Repair in the Adult CNS: the Central Role of Microglia,* p.19).

Microglia also interact specifically with synapses in the developing brain. One study addressed the developmental interactions of microglia and synapses by examining the visual cortex critical period using a (CX_3CR1+EGFP/Thy-1 YFP) transgenic mouse [98]. A number of features of visual perception are established during this period in mice, and are associated with well-described changes in dendritic spines [99-102]. This study showed that dendritic spines contacted by microglia can change size, and were often eliminated. In response to re-exposure to light following dark adaptation during this developmental period, microglia phagocytosed more synaptic elements compared with dark-adapted controls. This data suggests that in response to visual experience, spine elimination/remodelling is mediated at least partially by microglial phagocytosis [98, 102]. Microglia have been shown to directly phagocytose developmental synapses that express C1q via complement receptors on microglia; losing C1q expression in the mouse brain results in synapse elimination defects including a failure to refine synaptic connections [103]. In a more recent study, Stevens' group showed that developing retinal ganglion cell presynaptic terminals are pruned in a complement-dependent manner [104, 105]. Paolicelli and colleagues used stimulated emission depletion (STED) microscopy to demonstrate that post-synaptic density protein-95 (PSD95) immunoreactivity was present within microglial processes in the mouse hippocampus during normal development, suggesting that microglia can uptake pre- or postsynaptic material using clathrin and non-clathrin coated vesicles [106, 107]. An activity-dependent physical interaction between neurons and microglia, mediated by classical immune molecular activity, appears to refine synaptic connections during development.

At a network level, microglia-synapse interactions exert a real effect in development. CX3CR1 is expressed by microglia [108, 109]; its ligand fracktalkine (CX3CL1) can be either secreted by neurons or found at the cell surface, establishing an interaction between microglia and neurons in both the healthy and pathological CNS [110, 111]. CX3CR1 knockout mice display transient deficits in synaptic pruning, consistent with observations wherein single-cell recordings of CA1 pyramidal neurons revealed a decreased

sEPSC/mEPSC amplitude ratio, indicating immature connectivity in knockout animals [106]. In a follow-up study, the authors quantify the effect of this pruning deficit on synaptic transmission, functional connectivity and behavioural outcomes in mice, suggesting that a repetitive behavioural phenotype is reminiscent of neurodevelopmental disorder in humans [112]. As previously mentioned, neurodevelopmental disorders have been shown to associate with microglia-related neuroinflammatory changes, however CX3CR1 deficit has specifically been shown to impair learning, consistent with a substantial role for microglia in normal synaptic regulation and network development [113-117]. The possibility that defects in microglia function could lead to diverse developmental neuropathologies is an exciting future avenue for a multitude of therapeutic targets.

Pathology and Homeostasis in the Adult Central Nervous System

Maintenance of microglia populations, microgliosis, and parenchymal precursors

We are now beginning to understand how microglia are maintained in the adult brain and the factors responsible for regulating their population. It would appear that microglia are the only type of tissue-resident macrophages which maintain their population without contribution from the periphery throughout life [33, 61, 118, 119]. Colony-Stimulating Factor 1 (CSF-1) maintains macrophage populations through its protein tyrosine kinase receptor, CSF-1 receptor (CSF1R), which is expressed on committed macrophage precursors, monocytes, and tissue macrophages including microglia [33, 120, 121]. The osteopetrotic mouse is effectively CSF-$1^{-/-}$, causing a myriad of skeletal and haematopoietic defects including a reduction in microglia, which ranges from 30% to complete depletion depending on the region [120, 122-124]. Mutations in the CSF1R gene can cause even more severe defects, including a >99% microglia reduction brain-wide [125]. Elmore and colleagues found that blocking CSFR1 with PLX3397, a multi-targeted tyrosine kinase inhibitor, depleted adult mice of 99% of microglia, reinforcing the importance of CSFR1 signaling in microglia maintenance.

In a large functional screen of the human extracellular proteome, Lin *et al.* identified Interleukin-34 (IL-34), a previously unknown ligand for CSF1R [126]. IL-34 is more highly (approximately 10-fold) and broadly expressed compared to CSF-1 and their differential regional expression accounts for differences between CSF-1 and CSF1R knockout phenotypes [124]. Using IL-34 deficient mice, it was determined that IL-34 is a critical factor for microglia differentiation, with neurons representing the main sources of IL-34 [127]. In the adult mouse brain, CSF-1 and IL-34 are maintained at high levels, while CSF1R levels decrease during postnatal development [120]. A candidate alternate receptor for IL-34 has been identified indicating at least the possibility of differential functions of IL-34 and M-CSF [128]. More detailed information regarding colony stimulating factors and myeloid biology are reviewed in [129]. Whether IL-34 and M-CSF have differential influences on human microglial function is currently of interest.

Following brain injury microgliosis can occur: microglia numbers increase significantly in tissue proximal to the injury locus where they exhibit an activated morpohology and phenotype, increase expression of MHCII/HLA-DR [55], and inducible nitric oxide synthase (NOS2, iNOS) [57]. In the inflamed brain, the lack of specific markers capable of distinguishing circulating and invading peripheral blood-derived macrophages from microglia in histological sections precludes making completely accurate distinctions between these cell types. Initially, irradiation and bone marrow reconstitution experiments using congenic mice were used to examine the peripheral and central contributions to the microglia pool under various conditions, however, this model system generated various confounds [130]. In parabiosis, syngeneic mice have their circulatory systems joined such that host or donor cells can be tracked using specific markers. Thus, the host microglia population can be distinguished from peripheral cell types under both steady-state conditions and following experimental manipulations. Using this method, it was observed that blood-derived cells did not contribute to microglia turnover under steady-state conditions, nor microgliosis in models employing facial nerve axotomy, or overexpression of a transgenic mutant superoxide dismutase 1 ($SOD1^{G93A}$) [118]. Using a similar design but relying on a more controlled, modified irradiation-reconstitution method, Mildner and colleagues reported no participation of blood-derived cells in homeostatic microglial turnover, or in experimentally induced microgliosis (facial axotomy, and cuprizone-induced demyelination models) [119].

An understanding of the molecular factors regulating microglia population dynamics at a mechanistic level is developing. These will not be discussed in detail as they are reviewed sufficiently elsewhere [131]. Proliferation regulation signals include positive regulation via CLIC-1 and volume-sensitive chloride channels, adenosine receptors, and cannabinoid receptors [132-139]. Negative regulation may occur via voltage-dependent K_v channels, somatostatin receptors, glucocorticoid receptors, and beta-2 adrenergic receptors [140-143]. Albumin may cause microglia proliferation through intracellular Ca^{2+} signalling, providing an interesting candidate signal in the context of injuries that compromise blood brain barrier (BBB) integrity [131, 144]. Interestingly, a recent study found that the antiretroviral drug ganciclovir was a potent inhibitor of rodent microglial but not macrophage proliferation, indicating a differential proliferative mechanism *in vivo*

and the intriguing possibility of therapeutics selectively targeting microglial proliferation [145]. Now that we have identified selective molecular profiles for human and rodent microglia which can be used to distinguish them from macrophages, this may become a therapeutic possibility [67, 146, 147].

Despite the suggestion of local maintenance, little is known about microglia precursors in the adult brain. Nestin-positive microglia precursors were identified to re-populate the entire brain parenchyma following termination of treatment with PLX3397 within one week [148]. Nestin expression is upregulated in microglia following experimental injury, and can be found in many cell types near stroke infarcts, focal spinal cord injury, and in other injury models [149-154]. Whether erythromyeloid precursors express nestin, or whether this phenotype is indicative of a more mature precursor cell-type found in the brain alone remains to be answered. As a counterpoint, it is worth noting that no study has yet demonstrated a CNS-derived cell with stem characteristics that can generate microglia. Nestin-positive microglia precursors in Elmore *et al.* expressed Iba1, the lectin IB4, and CD45, with a fraction of cells also expressing Ki67, CD34 and c-Kit, but downregulated expression of these markers upon stabilization of their population [148]. In injury models, nestin expression following brain injury often occurs in cells of the perivascular region, indicating at least one putative niche for microglial precursors [155, 156]. Follow-up studies that isolate microglia precursors and meet the requisite criteria for *in vitro* dissociation/expansion and prove definitive stem characteristics will be required.

Neurobiology and Endogenous Repair in the Adult Central Nervous System

Microglia-synapse interaction in the adult brain

Since Kreutzberg popularized the facial axotomy model, it has been observed that microglia associate with injured nerve perikarya during synaptic stripping [157, 158]. Although some have described a potential role for glia-derived ATP or astrocytes in synaptic stripping of motorneurons [159-161], others favor a model involving the direct apposition of microglia with neuronal perikarya and apical dendrites [157, 162-164]. Interestingly, the fate of particular synapse terminals appears to depend on the type of input, since Linda et al. found that GABA and glycine terminals were preferentially preserved following motorneuron axotomy, although both excitatory and inhibitory inputs were drastically reduced overall [160]. This may support a transition from information processing towards a mode of survival and repair, protecting injured neurons from over-activity and the secondary effects of excitotoxic molecules, thus helping recovery from injury. Candidate molecular mechanistic targets for the stripping response include the CX3CR1 signalling axis and MHC Class 1 molecules as reviewed in [164]. Using 2-photon imaging strategies in which microglia and neurons are fluorescently labeled, it has been shown that microglia under basal conditions survey their microenvironment for signals and clearance of debris using highly dynamic processes [165, 166]. Wake and colleagues showed that basal microglia processes make brief, direct contact with synaptic terminals at a rate of approximately once per hour [167]. The frequency of these homeostatic contacts is activity-dependent, and is reduced concomitantly with neuronal activity. In that study, ischemic insult increased the duration of contact drastically, and was associated with a disappearance of the presynaptic bouton due to engulfment by microglia. Whether this activity is absolutely required for elements of synaptic homeostasis is still unknown, but the mechanisms of this activity are now becoming clearer.

It is conceivable that microglia could engage in crosstalk with neurons and release neurotrophins [168-170] or other signaling factors that could regulate synaptic structure and

transmission, thereby engaging in and modulating synaptic homeostasis. Certainly, microglia have myriad receptors to respond to secreted neuronal products and microglia have the ability to modulate neuronal functions, supporting a bidirectional model of communication in the adult organism [131]. BDNF derived from microglia was shown to modulate the neuronal anion gradient underlying neuropathic pain, directly regulating synaptic function [171]. Parkhurst and colleagues recently and definitively showed that microglia depletion in the otherwise healthy rodent brain induced deficiencies in multiple learning tasks and a specific reduction in motor-learning dependent synapse formation. This was largely regulated by microglial BDNF, as depletion of BDNF from microglia reproduced the effects seen in microglia-depleted rodents [172]. In the context of AD, it was shown that β-amyloid stimulation of iNOS and reactive oxygen species production in microglia inhibited NMDA-receptor dependent long-term potentiation and provides further evidence for a diverse array of neuronal and synaptic activities that can be modulated by microglia [173]. Furthermore, the neuroimmune interface with classical neurobiological activities is not reserved for microglia alone, as they have been shown to regulate hippocampal neurogenesis and maintain spatial learning abilities in adulthood through interactions with T cells [174].

Neuronal Repair in the Adult CNS: the Central Role of Microglia

In the adult CNS, microglia are attractive candidate therapeutic cellular targets for augmenting brain repair. In theory, this involves investigating a means to reduce microglia-induced damage while also boosting pro-neurogenic and/or oligodendrogenic mechanisms that are known to exist in adult microglia. As they have been covered extensively elsewhere, we will not discuss the mechanisms involved in neuroinflammatory damage to CNS tissue, for review see [175-179].

In vitro, LPS-activated microglia can block neurogenesis in organotypic hippocampal slice cultures; microglia activated by IL-4 and IFN-γ stimulate neurogenesis. Butovsky *et al*. attributed the blocking activity of LPS-activated microglia to TNF, whereas the augmenting effect of IL-4 and IFN-γ was attributed to Insulin-like Growth Factor 1 (IGF-1) [180]. In a rat model, microglia were found to be pro-neurogenic to striatal neuroblasts

derived from the ipsilateral SVZ following ischemia [181]. Using allogeneic co-cultures of human microglia and NPCs, Liu et al. showed that co-cultures had higher survival and proliferation of both NPCs and microglia compared to monocultures [182]. Despite evidence that microglia have pro-neurogenic activity [183, 184], some groups have found that microglia can also have anti-neurogenic activities [73, 185, 186], highlighting the dual role of microglia during adult neurogenesis, reviewed in [187].

Oligodendrocyte precursor cell (OPC) differentiation can also be influenced by microglia, making microglia attractive targets for augmenting repair in the context of demyelinating diseases. In an *in vitro* model, Butovsky and colleagues demonstrated the differential effects of IL-4 and IFNγ treated microglia towards augmenting oligodendrogenesis [180]. In a follow-up study, elevated IFNγ conferred a phenotype that blocked oligodendrogenesis with an associated increase in TNFα and decrease in IGF-1. This phenotype could be reversed by IL-4, whereby IL-4 activated microglia injected into the CSF of EAE mice improved clinical symptoms and increased oligodendrogenesis in the spinal cord [188]. Another line of evidence that activated microglia influence NPC and OPC differentiation includes ischemia studies where activated microglia secretory products influence neurosphere differentiation with a bias towards oligodendrocytes and neurons [189]. Miron et al. found that M2 cells drive oligodendrocyte differentiation during CNS remyelination in a focal demyelination model [50]. In human gliomas, Sarkar et al. found that brain-tumor initiating cells (BTICs) sphere-forming capacity was markedly reduced by microglia and macrophages derived from non-glioma human subjects. This activity was seen to involve the induction of cell cycle arrest and differentiation genes in BTICs. The authors demonstrated that low concentrations of amphotericin B increased glioma-derived microglial production of IL-8 and MCP-1 which curbed autologous BTIC growth [190]. Together these studies demonstrate the potential for modulation of microglia properties to therapeutic ends.

Microglia in Multiple Sclerosis: Summary

Microglia are a central component of the MS disease process within the brain. They are the brain's resident macrophages; they possess macrophage-like inflammatory and phagocytic properties, as well as antigen-presenting functions [19, 20, 22, 23, 26, 27, 36-39, 191-196]. Microglia are so integrated with MS pathology that the histopathological definition of an active or inactive MS lesion is defined by the stage of microglial phagocytosis and digestion of myelin components [6]. Indeed, microglia are found in all MS lesions; acute, chronic , slowly-expanding active, and inactive lesions defined by histopathological analysis as well as reactive lesions defined by T2 abnormality on magnetic resonance imaging [6]. Microglia are also involved in diffuse damage throughout the normal appearing white matter, as they are associated with inflammatory perivascular cuffs distributed throughout the MS brain, where damage to neurons may occur independently of demyelination and immune activity within plaques [6]. Microglial involvement in global MS pathology is substantiated by studies using positron-emission topography ligand [^{3}H]PK11195, which labels microglia and other myeloid cells in the inflamed and MS brain [197-199].

Microglia-mediated damage to neurons and oligodendrocytes is thought to occur through dysregulation of normal phagocytosis programs, production of reactive oxygen and nitrogen species, and cytokine secretion. In addition to directly injuring neural cell types, microglia-derived soluble factors can influence adaptive immune cells and endogenous brain repair mechanisms. Microglia are generally thought to cause damage to neurons and oligodendrocytes in MS, but in toxin-induced demyelination models have been shown to have some beneficial effects on remyelination [179]. Some have suggested that microglia may influence the neurogenesis and oligodendrogenesis required for repair in the adult CNS via production of soluble factors as well as phagocytosis [1]. Indeed, remyelination is known to occur in the presence of microglia, and may even occur concomitant with demyelination in some cases [6]. Microglial influence on endogenous differentiation mechanisms can both support and interfere with neural repair in the adult organism, and in this respect, differential activities are often associated with particular microglia polarization phenotypes [36, 37, 50]. Thus microglia are

considered to have the ability to influence both damage and repair of CNS tissue through a multitude of mechanisms [179].

Fumarate Treatment for Multiple Sclerosis

Evidence for different types of immune system dysfunction in MS have led to specific development and use of therapeutic agents [8]. Most recently, researchers have become interested in molecules with anti-oxidant activity as therapies for MS. It appears that CNS lesions may be an environment which results in true hypoxia, oxidative stress, or a hypoxia-like environment called *virtual hypoxia*, resulting in damage to oligodendrocytes and neurons [6]. Indeed, a recently approved MS therapeutic called dimethyl fumarate (Tecfidera®, BG-00012, DMF) is reported to have both anti-inflammatory and anti-oxidative properties in rodent models as well as humans [200-202]. Dimethyl fumarate (DMF) is a molecule originally used for psoriasis treatment as a component of a preparation of esters of fumaric acid also containing monomethyl fumarate (MMF) and monoethyl fumarate. Currently, purified DMF tablets are taken orally by MS patients; they are thought to be metabolized to MMF in the proximal intestine before absorption [203-207]. Thus, MMF is thought to mediate the majority of the effects of DMF treatment. However, pharmacokinetic studies have not made use of MS patients, only healthy controls, psoriasis patients, and mice. Moreover, studies have shown significant DMF-specific metabolic breakdown products in the urine of psoriasis patients making DMF interesting for researchers interested in mechanism of action, particularly in the context of chronic inflammatory diseases. Thus, most studies employ the use of DMF treatment *in vivo* to mimick Tecfidera® treatment in humans, and DMF and MMF treatment *in vitro* to elucidate the cellular effects of either drug species separately.

Both DMF and MMF are thought to have similar chemical activity with the overall potency of DMF being greater than that of MMF. There is current interest in other compounds with similar chemical activity profiles, namely thioreductive activity, in this case produced via the α,β-unsaturated structure of DMF and MMF [208]. DMF/MMF pharmacodynamics related to their mutual thioreductive capacity include direct extracellular glutathione depletion and NRF2 induction by covalent binding to cysteine residues on the cytosolic inhibitor, KEAP1, inducing its degradation. Overall, this results in the induction of genes containing antioxidant response elements (AREs) in their promoter region such as HMOX1, NQO1, and OSGIN1. Given the mechanistic

generality implied by such a thioreductive activity, it is also likely that the function of many so-called cysteine-sensing proteins may be altered by DMF or MMF treatment and corresponding transcriptional programs modified. However, the cysteine-sensing redox signaling system is a novel mechanistic axis in the context of ongoing stress and inflammation such as exists in MS; little can be said about this complex system in the context of overall pathology at this time. Currently, thioreductive activity has been reported in a number of compounds such as supercumin which demonstrate some anti-inflammatory activity in similar cellular contexts [208]. The structures of dimethyl fumarate (A) and monomethyl fumarate (B) are shown in **(Fig. 2)**.

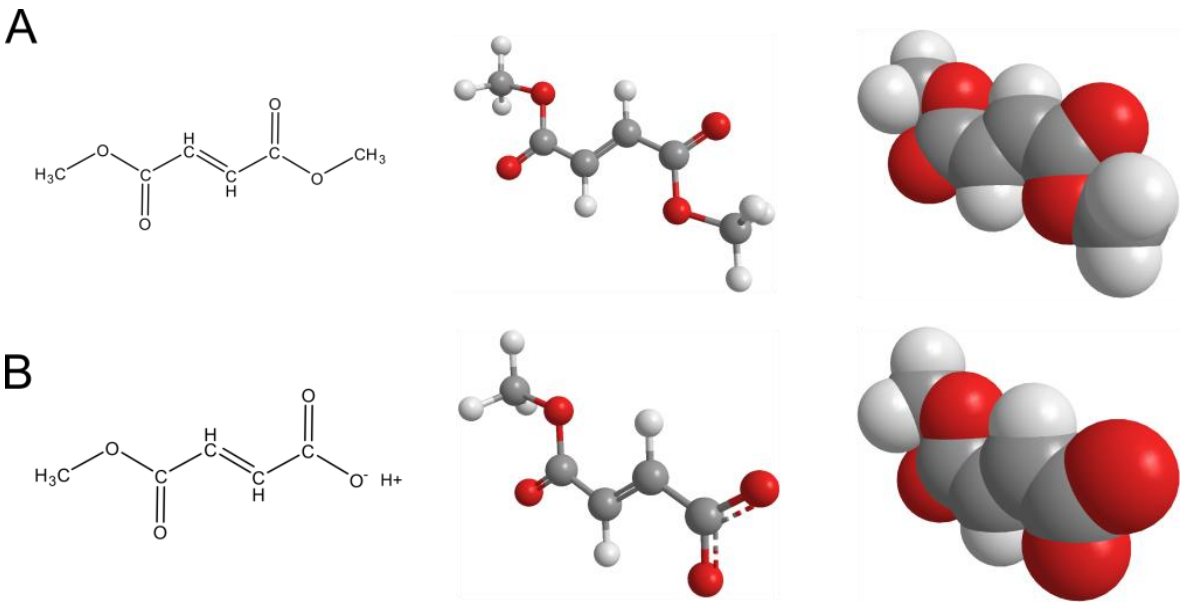

Figure 2 – Lewis, ball and stick, and space-filling structure models of Dimethyl fumarate (A) and Monomethyl fumarate (B). Structural images created using ChemBioDraw Ultra Suite (Cambridgesoft, 2015).

Specific cellular mechanisms for either DMF or MMF are not to be ignored. Several studies have implicated Hydroxycarboxylic Acid Receptor 2 (HCAR2, GP109A), the specific receptor for MMF in rodent cells, which is thought to mediate at least some of the effects of treatment in humans. For instance, the flushing and diarrhea experienced by the majority of patients during the onset of treatment are also observed during niacin treatment for hyperlipidemia, a specific ligand for the HCAR2 receptor on immune cells and adipose tissue. DMF treatment in rodent EAE models was found to reduce the infiltration of neutrophils in an HCAR2-dependent manner. Whether DMF itself or DMF-

related metabolites have specific receptors in the human body and whether this is relevant to human pharmacodynamics remains to be clarified. That said, the presence of specific DMF and MMF associated genetic profiles and differential cellular effects in ours and others work indicates the potential for specific mechanisms underlying the activities of each drug.

PART 3: THE EFFECTS OF FUMARATE TREATMENT ON MYELOID CELLS IN MULTIPLE SCLEROSIS

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Effects of fumarates on circulating and CNS myeloid cells in multiple sclerosis

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Abstract

Objective: Dimethyl fumarate (DMF), a therapy for relapsing-remitting multiple sclerosis (RRMS), is implicated as acting on inflammatory and anti-oxidant responses within either the systemic immune or CNS compartments. Orally administered DMF is rapidly metabolized to monomethyl fumarate (MMF). Our aim was to analyze the impact of fumarates on the anti-inflammatory and anti-oxidant profiles of human myeloid cells that are found in the systemic compartment (monocytes) and in the inflamed CNS (blood derived macrophages and brain derived microglia).

Methods: We analyzed cytokine and anti-oxidant expression in monocytes from untreated or DMF-treated RRMS patients and controls, and in monocyte-derived macrophages (MDMs) and microglia isolated from adult and fetal human brain tissue.

Results: Monocytes from MS patients receiving DMF had reduced inflammatory responses (miR-155 expression) and reduced expression of anti-oxidant genes HMOX1 and OSGIN1 compared to untreated MS patients; similar changes were observed in patients receiving FTY720. *In vitro* addition of DMF but not MMF to MDMs and microglia inhibited lipopolysaccharide-induced production of inflammatory cytokines and increased expression of the anti-oxidant gene HMOX1 without upregulation of NQO1 in absence of significant cytotoxicity.

Interpretation: Our *in vivo*-based observations that effects of DMF therapy on systemic myeloid cell inflammatory and anti-oxidant gene expression are also observed with FTY720 therapy suggests that the effect may be indirect, reflecting reduced overall disease activity. Our *in vitro* results demonstrate significant effects of DMF but not MMF on inflammation and anti-oxidant responses by monocyte-derived macrophages and microglia, questioning the mechanisms whereby DMF therapy would modulate myeloid cell properties within the CNS.

Introduction

Multiple sclerosis (MS) often manifests clinically as a relapsing disorder that evolves to take a progressive course. The activation and entry of peripheral immune cells into the central nervous system (CNS) is thought to initiate lesion formation. However, a compartmentalized immune response within the CNS is considered to sustain the initial inflammatory response and contribute to subsequent evolution into the progressive phase of the disease. Members of the innate immune system in both the peripheral and CNS compartments are implicated as contributors to all phases of the disease. Peripheral blood-derived monocytes have a pro-inflammatory bias in both relapsing and progressive-course patients [209, 210]. Within the CNS, myeloid cells are comprised of both brain-resident microglia and infiltrating macrophages, and show an activated, pro-inflammatory phenotype.

The mechanisms underlying the clinical efficacy of dimethyl fumarate (DMF) therapy for MS require further study. Pharmacokinetic studies in healthy subjects indicate that conversion of DMF to its primary metabolite monomethyl fumarate (MMF) occurs prior to entering the circulation [204, 207]. However, studies of psoriasis patients demonstrate that specific breakdown products of DMF can be detected in urine, indicating the potential for absorption of DMF in chronic inflammatory conditions [211]. Hydroxycarboxylic Acid Receptor 2 (*GP109A,* HCAR2) is recognized as a high affinity receptor for MMF [212, 213].

DMF therapy decreases the severity of experimental autoimmune encephalomyelitis; this effect is reduced signficantly in animals with genetic deletions of the Nuclear Factor (Erythroid-Derived 2)-Like 2 (*NFE2L2*, NRF2) [214, 215], a regulator of anti-oxidant responses. Nrf2 is inhibited by Keap-1 under steady state conditions but is released and translocates to the nucleus in response to cellular oxidants [214, 216, 217]. *In vitro* studies implicate effects of either MMF or DMF on anti-oxidant responses, as well as on inflammation-related responses. Linker et al. showed that MMF can bind cysteine residues on Keap-1 leading to activation of Nrf2 [214]. Linker et al. and Scannevin et al. also found that DMF and MMF increased NRF2 activity and reduced susceptibility to

oxidative stress in astrocytes *in vitro*; the latter effect was linked to up-regulation of anti-oxidant genes including HMOX-1 and NQO1 [214, 215]. MMF is reported to induce tumor necrosis factor (TNF) and IL10 in human monocytes *in vitro* [218]. However, Lehman et al. found that only DMF decreased inflammatory cytokine production in PBMCs [219]. DMF is also shown to decrease TNF and IL-6 mRNA in rat microglia *in vitro* [220]. The anti-oxidant and inflammation related effects may be interrelated since the antioxidant gene HMOX1 can act as a regulator of inflammation [221-223].

Our aim was to analyze the impact of DMF and MMF on the anti-inflammatory and anti-oxidant profiles of human myeloid cells including CD14+ monocytes, monocyte-derived macrophages, and brain-derived microglia. We present data demonstrating *in vivo* effects of DMF therapy on these responses in monocytes of MS patients, but also observe similar effects with FTY720 therapy. We observe *in vitro* effects of fumarate therapy including on CNS compartmentalized myeloid responses (macrophages/microglia) with DMF rather than MMF. Our combined *in vivo* and *in vitro* observations raise issues with the mechanisms currently thought to underpin the efficacy of this therapy in MS.

Methodology

Subjects and recruitment

Peripheral blood samples were collected into K_2EDTA-coated plastic tubes from a total cohort comprised of healthy subjects (n = 22, mean age 45 years, 15 female) and MS patients who were either untreated (n = 27, mean age 41 years, 18 female) or were receiving DMF (n = 32, mean age 43 years, 23 female) or FTY720 (n = 16, mean age 44 years, 10 female). None of the MS patients had clinical relapses within three months of study. All study details were approved by the institutional review board at McGill University in accordance with Canadian Institutes of Health Research guidelines and all subjects provided informed consent.

Quantitation of blood cell populations

Complete blood counts were obtained retrospectively for 24 DMF-treated patients from our patient information database. Patients with significant lymphopenia would not have been included in the study. On average, pre-treatment counts were obtained 212 days prior to the treatment start date (standard deviation of 256 days). On average, post-treatment counts were obtained 185 days following initiation of treatment (standard deviation of 108 days).

Cell Culture (Human monocytes, macrophages, and microglia)

PBMCs were isolated from whole blood using Ficoll-Pacque density gradient centrifugation (GE Healthcare, Baie-d'Urfé, Quebec, Canada). CD14+ cell isolation was done using CD14 immunomagnetic bead selection according to manufacturer's protocols to 95-98% purity (Miltenyi Biotech, Auburn, CA). Monocytes were either lysed immediately in Trizol reagent or cultured at $5x10^5$ cells mL^{-1} in RPMI media supplemented with 10% FBS, 0.1% penicillin/streptomycin (P/S) and 0.1% glutamine in sterile, loose-capped, 5 mL polypropylene tubes. Monocyte-derived macrophages (MDMs) were differentiated *in vitro* by culturing at $5x10^5$ cells mL^{-1} in 10% RPMI with 25 ng mL^{-1} M-CSF for five days in 6 well culture plates as described [36].

Human microglia were isolated from either fetal or adult brain tissue using previously described protocols [36, 224]. Briefly, brain tissue was mechanically dissociated and underwent enzymatic digestion using trypsin and DNAse prior to mechanical separation through a nylon mesh filter. Adult tissues underwent an additional ultracentrifugation step to remove myelin. Dissociated cells were then centrifuged, counted, and plated at either $6x10^6$ cells mL^{-1} in DMEM with 5% FBS and 0.1% P/S, and 0.1% glutamine (fetal) or $2x10^6$ cells mL^{-1} in minimum essential media with 5% FBS and 0.1% P/S, and 0.1% glutamine (adult). Microglia were grown for 10-14 days with one media replacement after 5-7 days in culture. Purified microglia are then collected and plated at $1x10^5$ cells mL^{-1} and maintained in culture for 5 days before treatments. For experiments involving HCAR2 expression, astrocytes isolated from the fetal human tissue specimens and cultured as described in [225] were included as controls.

In vitro drug treatments

DMF and MMF (stock concentrations of 2mM, Sigma Aldrich) aliquots were prepared on the same date and frozen at -80°C with aliquots of vehicle (DMSO). Our initial *in vitro* work indicated that DMF obtained from Biogen Idec had identical effects to DMF from Sigma Aldrich that was used for the study. Cells were pre-treated with either compound 1-hour prior to the addition of 100 ng mL^{-1} lipopolysaccharide (LPS). LPS stimulation was carried out for either 6 or 24 hours as indicated before supernatant collection, live /dead cell analysis, or RNA extraction procedures.

Quantitation of gene and miRNA expression by quantitative, real-time polymerase chain reaction

Collected cells were lysed in Trizol reagent and stored at -80°C. Total RNA extraction was performed using standard Trizol protocols followed by DNAse treatment according to manufacturer's instructions (Qiagen, Valencia, CA). For gene expression analysis, standard reverse transcription (RT) using random hexaprimers and moloney murine leukemia virus reverse transcriptase was done. For miRNA expression analysis, multiplexed RT reactions were performed using a mix of miRNA-specific RT primers and a TaqMan® MicroRNA RT kit (Life Technologies). Individual gene expression or

miRNA expression assays were performed using specific TaqMan® or miRNA TaqMan® probes to assess expression relative to 18s or RNU48, abundant and stable housekeeping RNAs for gene and miRNA expression analysis, respectively. Fold Change calculations were performed using the -ΔΔCT method.

Quantitation of cytokine secretion by enzyme-linked immunosorbance assay (ELISA)

Cell culture supernatants were collected following *in vitro* experiments and stored at -80°C. ELISAs for TNF, IL-6 and IL-10 were performed in duplicate following manufacturer's protocols (BD Biosciences, Mississauga, ON).

Live/dead cell assays

Flow cytometry based - for these assays, healthy and patient monocytes were incubated with either DMF or MMF (1 – 50 μM) ± LPS *in vitro* for 24 hours. Cells were then incubated with 0.2 μM Calcein AM for 20 minutes at 37°C and 5% CO_2 before a single wash in FACS buffer, and application of 0.5 μM propidium iodide (PI) for 15 minutes prior to acquisition using an LSR Fortessa flow cytometer (BD Biosciences).

Fluorescent microscopy - for these assays 4 μM Calcein AM and 1 μM propidium iodide were added to wells of monocyte, MDM, and microglia cultures that had been exposed to DMF or MMF for 20 minutes at 37°C and 5% CO_2. Following incubation, cells were washed twice in PBS and directly imaged using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). For the green and red channels, an exposure of 90 and 880 milliseconds were used respectively. 8-bit images from green and red fluorescence channels were background subtracted using a 50 pixel rolling-ball radius, and merged to create color composites using standard ImageJ functions (Bethesda, MD). Cells were counted manually and the observer was blinded to condition.

Live Cell Imaging - Microglia were plated directly onto the glass window of glass-bottom cell culture dishes (MatTek, Ashland, MA) and culture media was replenished further to a total of 2 mL after 24 hours. Cells were pre-treated with DMF or MMF at the indicated concentration for 20 minutes prior to imaging. Dishes were then moved to the incubator

attached to a VivaView live-imaging instrument (Olympus, Center Valley, Pennsylvania) and imaged each 12 minutes over six hours using differential interference contrast at 20X magnification. Images were compiled to create a movie using standard ImageJ functions (Bethesda, MD).

HCAR2 Expression on Microglia and Astrocytes

For quantitation of HCAR2 expression, microglia or astrocytes were detached and collected from six-well culture plates using 2mM EDTA in warm PBS for five minutes. Cells were blocked in 10% normal human serum and normal mouse immunoglobulin G (3 ng mL^{-1}) in flow-activated cell sorting (FACS) buffer (1% FBS in PBS) and then incubated with 0.1 ng anti-HCAR2 or isotype APC-conjugated antibody (R&D Systems) for 40 minutes, followed by one wash in FACS buffer. To confirm the identity of astrocytes, these cells were then permeabilized and fixed with 10% saponin and 1% formaldehyde, and further incubated with anti-GFAP or isotype alexa-488 conjugated antibody for 20 minutes. Microglia were selected using the side-/forward-scatter profile according to previously described methods [36, 37]. Events were acquired using an LSR Fortessa flow cytometer (BD Biosciences); debris and dead cells were gated out of analyses, as were doublets using forward scatter height versus width discrimination.

Results

Effects of DMF therapy on circulating monocytes

Overall numbers of circulating monocytes were within normal range for all the DMF treated patients in the study. However, as expected there was a trend for an on-treatment relative reduction in lymphocyte counts (mean 1.71 x 10^6 per mL^{-1} blood for pre-treatment counts versus 1.47 x 10^6 per mL^{-1} blood for post-treatment counts) and a relative increase in monocytes (mean 4.60 x 10^5 per mL^{-1} blood for pre-treatment counts versus 5.29 x 10^5 per mL^{-1} blood for post-treatment counts).

MiR-155 is an MS-relevant marker of inflammation in circulating monocytes [37]. We observed significantly lower levels of miR-155 expression in the monocytes of patients treated with DMF relative to monocytes from untreated RRMS patients **(Fig. 3A)**.

As regards anti-oxidant gene expression, we observed that HMOX1 expression was significantly higher in untreated MS patients relative to healthy controls (**Fig. 3B:** mean = 435.5 fold-induction, standard deviation = 133.6, p = <0.0001), followed by OSGIN1 **(Fig. 3C:** mean = 4.8 fold induction, standard deviation = 3.5, p = 0.0004); NQO1 was not elevated in untreated RRMS patients **(Fig. 3D)**. HMOX1 levels in DMF-treated patients were reduced compared to untreated MS patients but remained significantly elevated compared to healthy controls ($p < 0.0001$), DMF-treatment was also associated with decreases in OSGIN1 relative to untreated patients ($p < 0.0001$); levels in treated patients were not significantly different from healthy controls ($p = 0.4941$).

For both miR-155 and anti-oxidant gene expression, similar reductions as seen with the DMF treated cohort were also observed in patients receiving FTY720 when compared to untreated patients **(Fig. 3A to D)**.

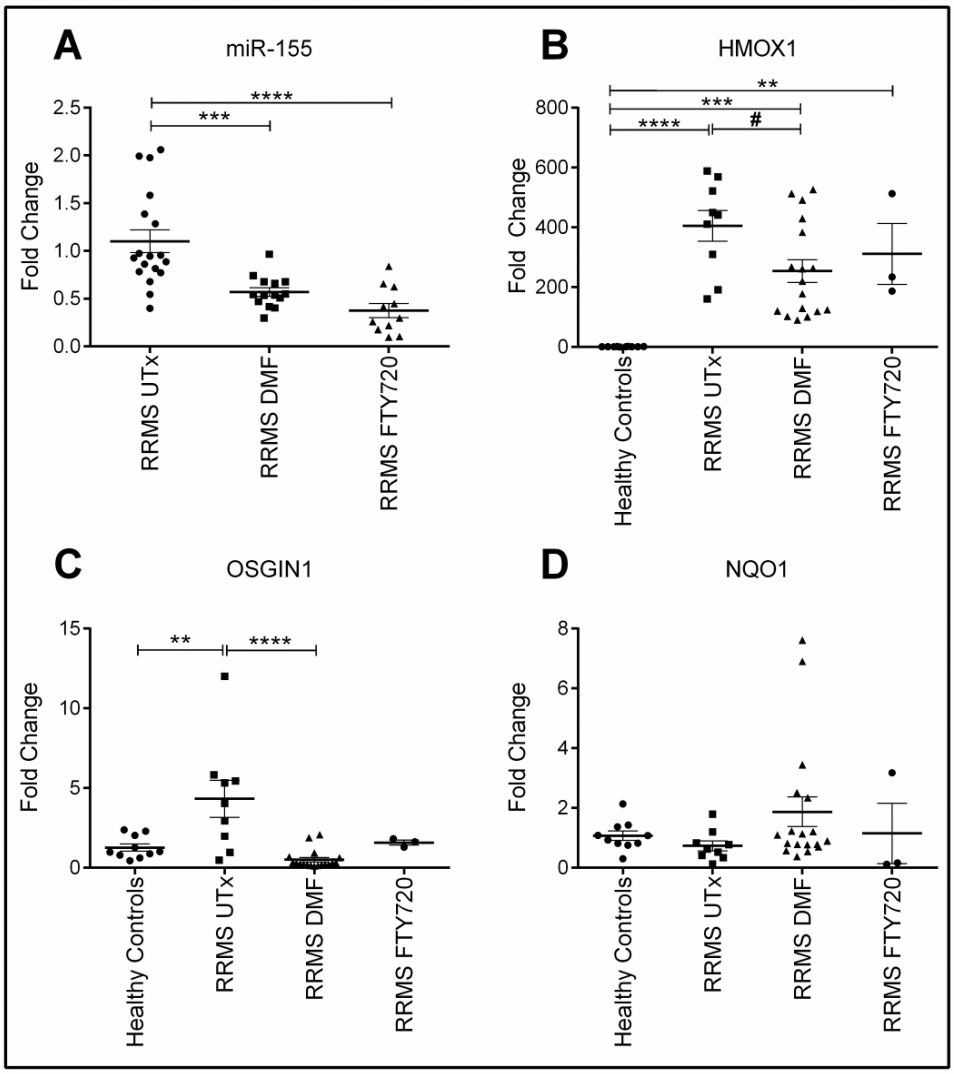
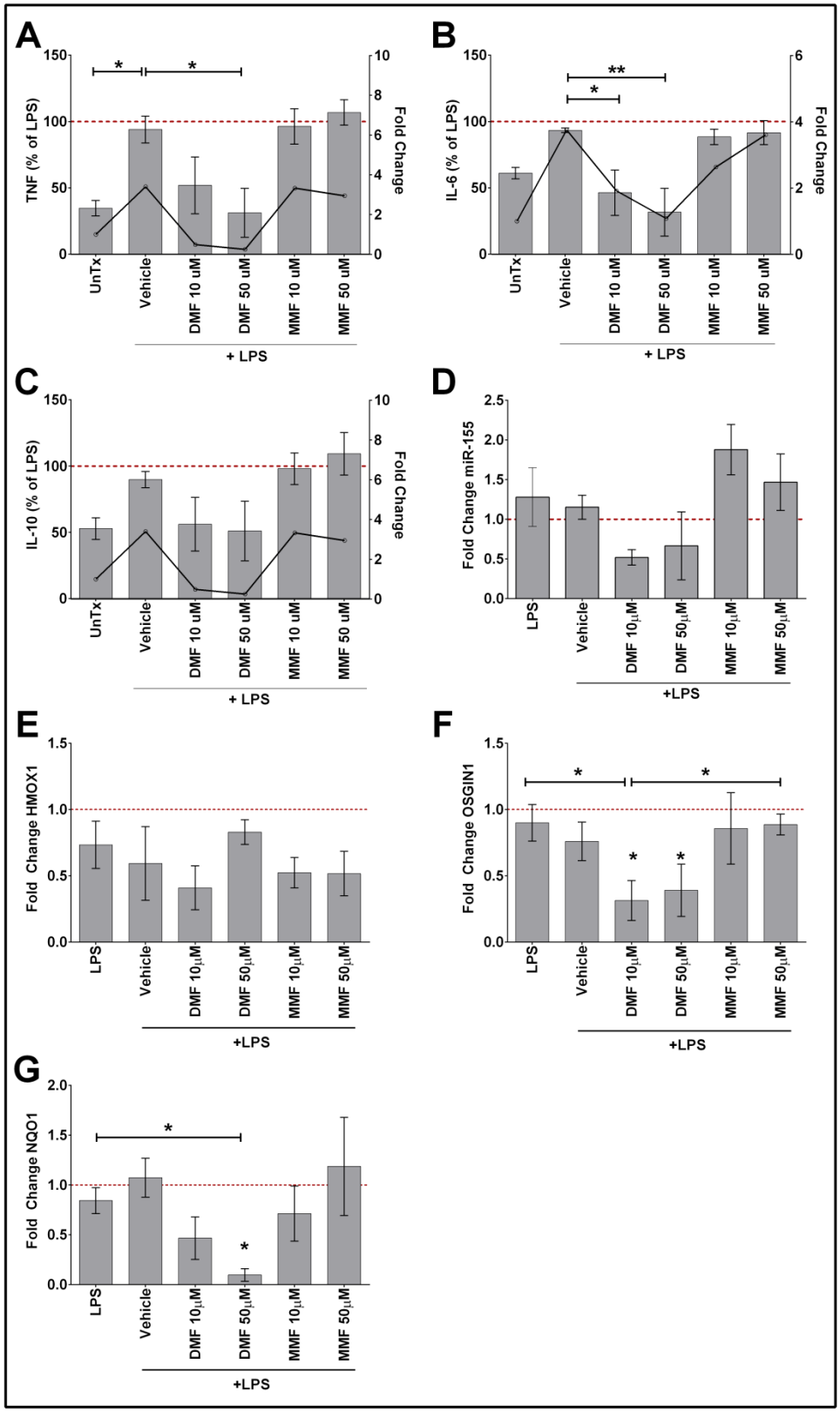


Figure 3: Anti-inflammatory and anti-oxidative effects of DMF treatment in monocytes isolated from RRMS patients. (A) DMF-treated and FTY720-treated patients were observed to have reduced miR-155 levels compared to untreated RRMS patients. Data are expressed as fold change relative to healthy controls. (B-D) HMOX1 and OSGIN1 but not NQO1 expression was significantly higher in untreated MS patients relative to healthy controls. HMOX1 and OSGIN1 expression levels were reduced in DMF and FTY compared to untreated MS patients. Levels of HMOX1 remained increased in comparison to healthy controls, whereas OSGIN1 stabilized to healthy control levels. Similar levels of miR-155, NQO1, HMOX1, and OSGIN1 were observed in monocytes from patients treated with DMF and FTY720. Each data point indicates a single individual. One-way ANOVA compares means of all groups against each other, $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. # Comparison using ANOVA against Untreated MS patients alone yields significant difference between these two groups (P=0.0293). Thanks to Dr. Craig Moore for FTY720 patient miR-155 data points in 3A, included with permission.

Effects of DMF and MMF on CD14+ monocytes *in vitro*

We initially observed that DMF added *in vitro* inhibited LPS-induced TNF**,** IL-6, and IL-10 expression in monocytes at the protein and mRNA level **(Fig. 4 A to C)**. DMF treatment was also associated with a non-significant decrease in basal miR-155 **(Fig. 4D);** miR-155 was not significantly regulated by LPS alone. Anti-oxidant gene expression (NQO1 and OSGIN1, but not HMOX1) was decreased in the presence of DMF **(Fig. 4 E to G)**.

For these studies, monocytes were maintained in vitro for up to 24 hours. At 24 hours mean cell death of monocytes from healthy control donors under basal conditions was 11.3% (standard deviation 8.7, n=5). No significant differences in cell death were observed between treated MS, untreated MS, and healthy control donor monocytes cultured under basal conditions. We further observed that DMF, when added to monocytes *in vitro,* significantly increased levels of cytotoxicity from baseline values. This occurred in a dose-dependent manner, first documented by trypan blue exclusion at six hours (data not shown), and subsequently by counting PI-positive cells using flow cytometry following exposure to DMF for 24 hours *in vitro* **(Fig 5)**. 10 μM DMF added to monocytes in basal media ($p<0.05$) and 50 μM DMF added to LPS stimulated monocytes ($p<0.01$) were cytotoxic after 24 hours *in vitro*. We found no differences in levels of toxicity between monocytes from DMF treated patients and healthy controls by regular two-way ANOVA. MMF had no significant effects on any of the above cytotoxicity measures.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DMF 50μM
MMF 10μM
MMF 50μM
+LPS
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F
Fold Change OSGIN1
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Fold Change NQO1

Figure 4

Figure 4: DMF but not MMF treatment *in vitro* reduces LPS-induced cytokine production and anti-oxidant gene expression in monocytes (p. 37). Monocytes isolated from peripheral blood mononuclear cells of healthy controls were pre-treated for one hour with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for six hours. (A-C) DMF inhibits LPS-induced TNF, IL-6, and IL-10 protein production (grey bars) and mRNA expression (bold black line) in monocytes. Protein levels are expressed as percent of LPS-only condition (dotted horizontal red line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, error not shown (n=3); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Holm-Sidak multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; n=4. (D) DMF treatment *in vitro* was also associated with a non-significant decrease in miR-155; miR-155 was not significantly regulated by LPS alone. (E-G) Anti-oxidant gene expression (NQO1, OSGIN1) was decreased in presence of DMF. Grey bars indicate mean RNA level versus untreated condition (dotted horizontal red line). Repeated measures one-way ANOVA; Tukey's multiple comparisons test compares all columns; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; n=3.

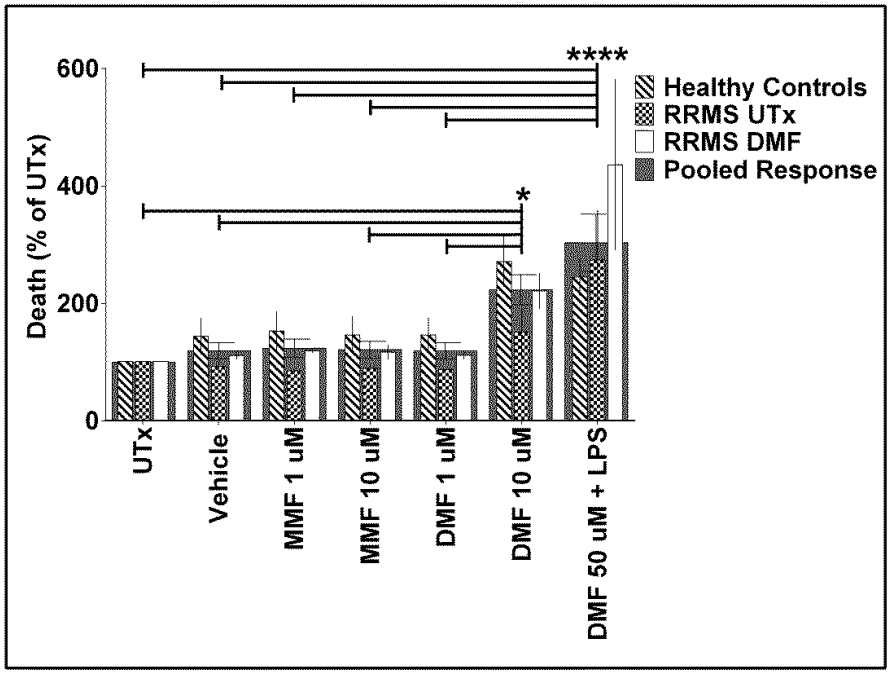

Figure 5: DMF *in vitro* is cytotoxic to monocytes from healthy controls and patients (p.38). Monocytes were cultured immediately following positive selection with anti-CD14 immunomagnetic beads in the presence of low dose DMF and MMF (1 and 10 μM), as well as 50 μM DMF and LPS (positive control), and analyzed via PI staining and flow cytometry. Both 10 μM DMF ($p<0.05$) and 50 μM DMF + LPS treatment ($p<0.01$) induced significant cytotoxicity after 24 hours. No toxicity was observed with MMF. One-way ANOVA with Tukey's multiple comparisons test compares means of pooled response group. There was no differential cytotoxicity of monocytes derived from untreated or DMF-treated patients, or healthy controls as assessed by regular two-way ANOVA with Tukey's multiple comparisons test. Healthy Controls n = 4, RRMS Utx n=3, RRMS DMF n=3; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

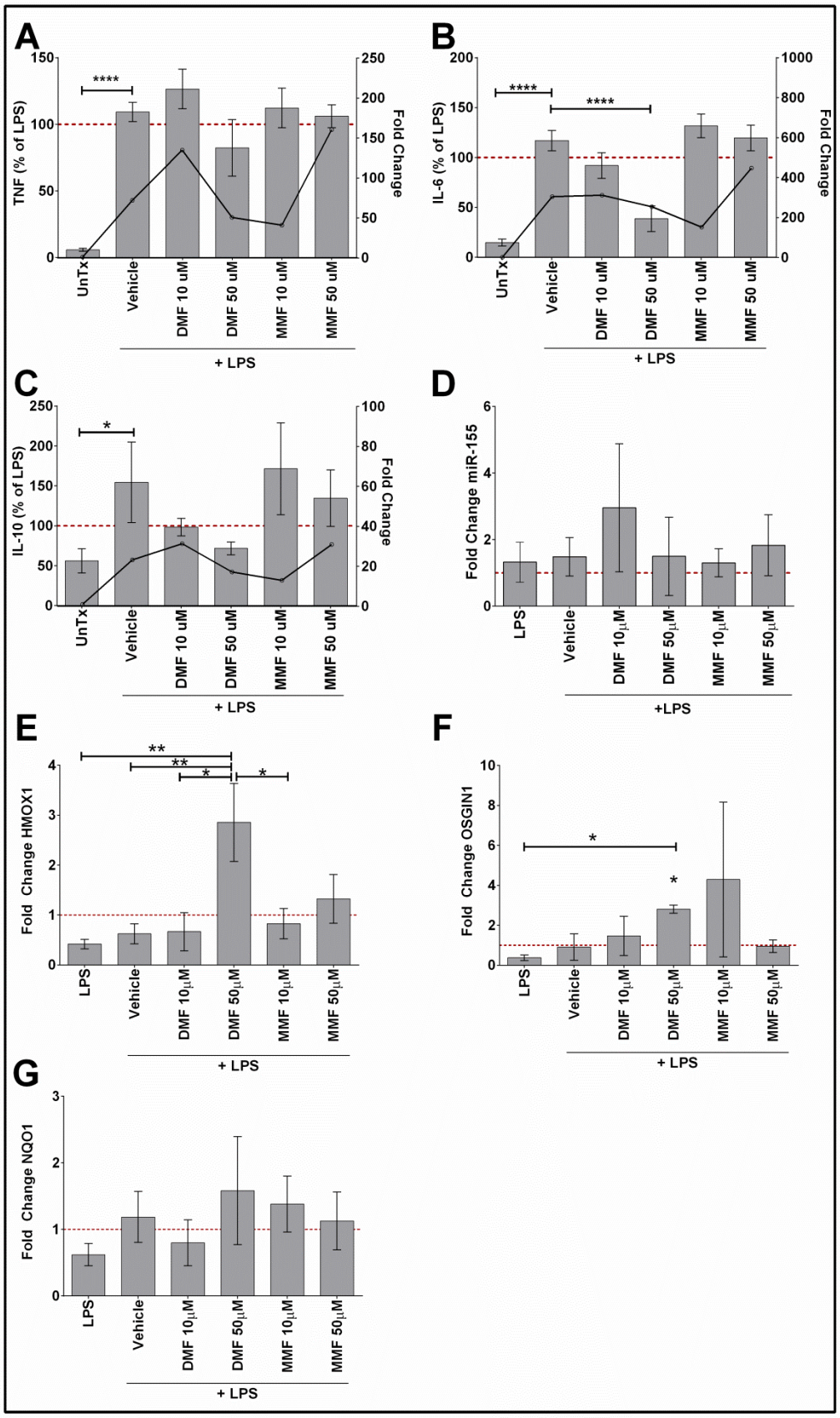
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Figure 6

Figure 6: DMF treatment *inhibits* LPS-induced IL-6 and *induces* HMOX-1 expression in monocyte-derived macrophages (MDMs) in absence of cytotoxicity (p.40). MDMs from healthy controls were pre-treated for one hour with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for six hours. **(A-C)** DMF-treatment produced down-regulation of IL-6 **(B)** but not of TNF **(A)** or IL-10 **(C)**. Protein levels are expressed as percent of LPS-only condition (dotted horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, error not shown (n=3); data are expressed as fold change relative to healthy controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; n=8. **(D)** miR-155 - no significant regulation of miR-155 in response to LPS, DMF or MMF (n=3). **(E-G)** HMOX1 **(E)** and OSGIN1 **(F)** were significantly upregulated by 50 µM DMF, while NQO1 **(G)** was not. Grey bars indicate mean RNA level versus untreated condition (dotted horizontal line). Repeated measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus each other; $\alpha = 0.05$; * n=7 except OSGIN1 n=3.

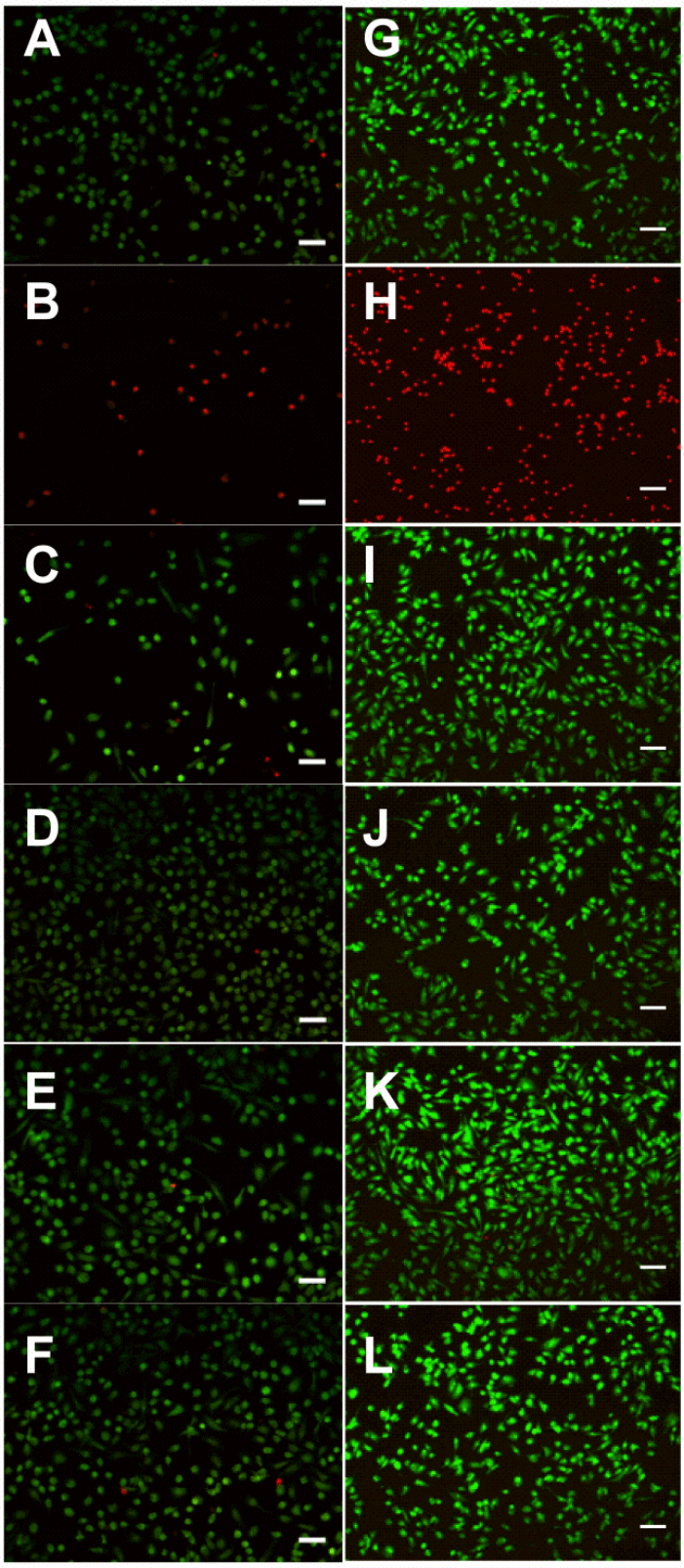
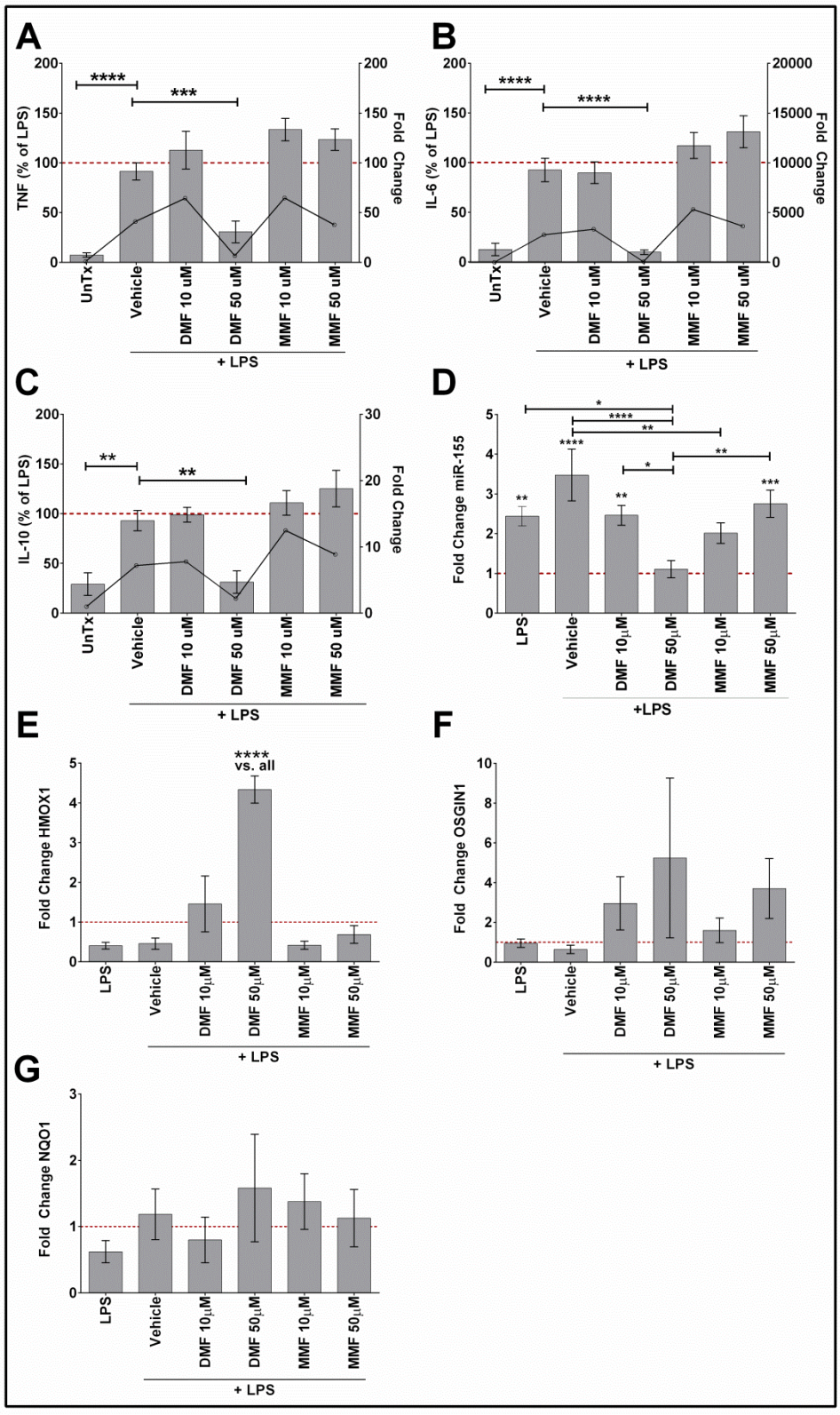


Figure 7

Figure 7: Neither DMF nor MMF is toxic to monocyte-derived macrophages or microglia *in vitro* (p.41). MDMs **(A-F)** and human fetal microglia **(G-L)** stained with viability marker Calcein AM (green) and death marker propidium iodide (red) show no cytotoxicity in response to 50 μM DMF and MMF. **(A)** Untreated MDMs **(B)** Positive Control: MDMs treated with 0.1% saponin during staining **(C)** Vehicle + LPS **(D)** LPS **(E)** DMF 50 μM + LPS **(F)** MMF 50 μM + LPS. Experiments were done in triplicate from three independent donors with identical results; representative experiment shown. These experiments were repeated in human fetal microglia with identical results (n=4, representative shown). **(G)** Untreated microglia **(H)** Positive Control: microglia treated with 0.1% Saponin during staining **(I)** Vehicle + LPS **(J)** LPS **(K)** DMF 50 μM + LPS **(L)** MMF 50 μM + LPS.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G
Fold Change NQO1

Figure 8

Figure 8: DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression and *induces* HMOX1 expression in human fetal microglia (p.43). Human fetal microglia were pre-treated for one hour with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for six hours. **(A-C)** 50 μM DMF but not MMF pre-treatment inhibits LPS induced TNF **(A)**, IL-6 **(B)** and IL-10 **(C)** production in fetal microglia. Protein levels are expressed as percent of LPS-only condition (dotted horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, error not shown (n=3); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; * $p<0.05$, ** $p <0.01$, *** $p<0.001$, **** $p<0.0001$; n=8. **(D)** 50 μM DMF pre-treatment of human fetal microglia inhibits LPS-induced miR-155 expression. Significance of changes in expression were assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns to each other; $\alpha = 0.05$; * $p<0.05$, ** $p <0.01$, *** $p<0.001$, **** $p<0.0001$; n=4. **(E-G)** DMF induced up-regulation of HMOX1 **(E)**, but not OSGIN1 **(F)** or NQO1 **(G)** in human fetal microglia. Grey bars indicate mean RNA level versus untreated condition (dotted horizontal line). One-way ANOVA with Tukey's multiple comparisons test compares all columns versus each other; $\alpha = 0.05$; * n=5 except OSGIN1 n=4.

Effects of fumarates on MDMs

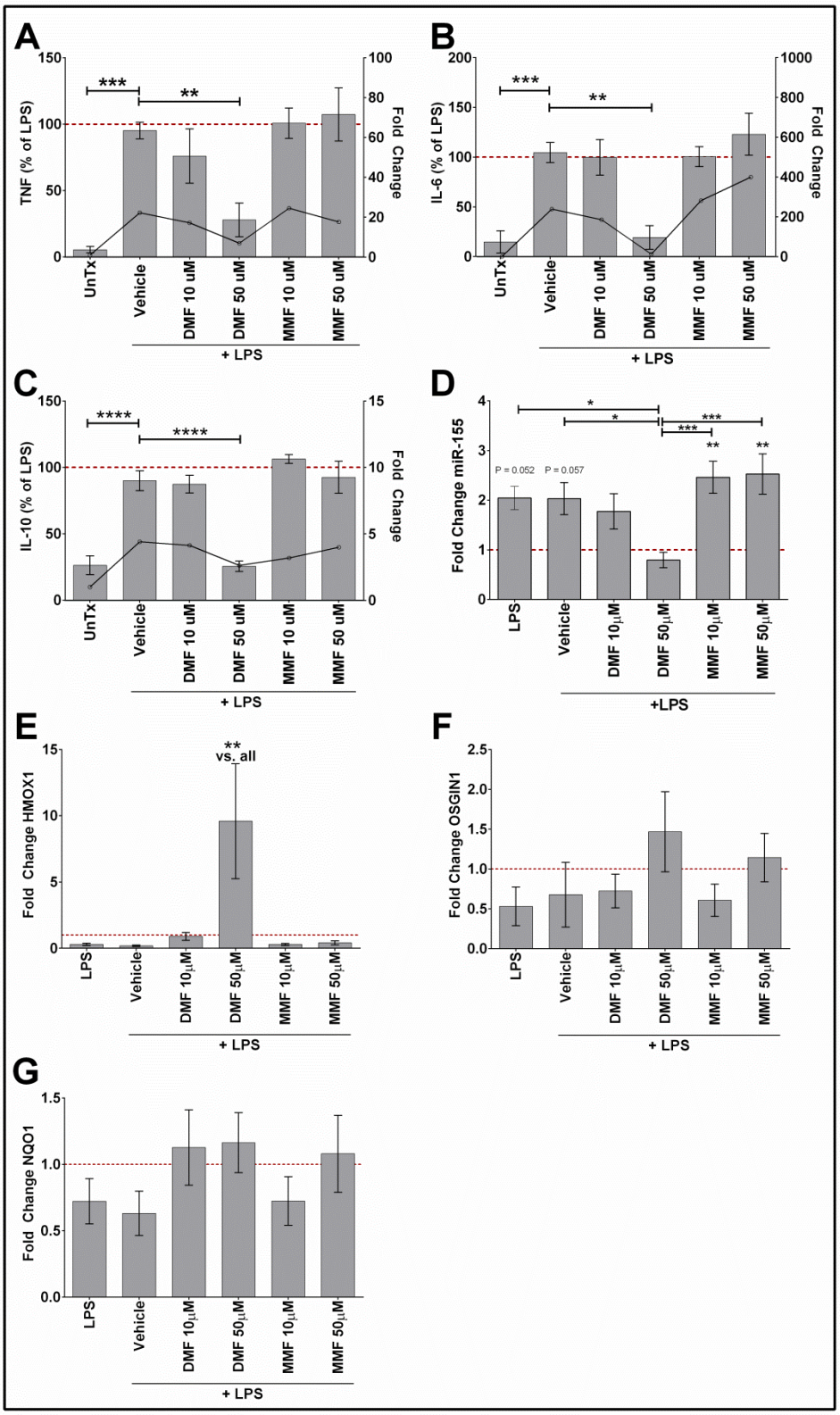
When we tested DMF and MMF on MDMs *in vitro* we observed a significant DMF-associated down-regulation of IL-6 **(Fig. 6B)** but not of TNF **(Fig. 6A)** or IL-10 **(Fig. 6C)**. HMOX1 **(Fig. 6E)** and OSGIN1 **(Fig. 6F)** were significantly upregulated by 50 μM DMF. There was no significant regulation of miR-155 **(Fig. 6D)**, or NQO1 **(Fig. 6G)** in MDMs. We did not observe any effect of MMF on these functional response measures. We did not observe cytotoxicty to MDMs under any of the conditions tested **(Fig. 7A to F)**.

Effects of fumarates on adult and fetal microglia

Overall, we observed that microglia were more sensitive to inhibition of cytokine and induction of HMOX1 by DMF treatment compared to peripheral myeloid cells. We observed significant inhibition of LPS-induced TNF**,** IL-6, and IL-10 **(Fig. 8 A to C)** production in human fetal microglia pre-treated with 50 μM DMF, but not MMF. Pre-treatment with 50 μM DMF also inhibited LPS-induced miR-155 production in human fetal microglia **(Fig. 8D)**. Similarly, we observed inhibition of TNF, IL-6, and IL-10 by 50 μM DMF in human adult microglia (**Fig. 8A to C)**. Levels of miR-155 expression in LPS treated human adult microglia were decreased in the 50 μM DMF pre-treatment condition **(Fig. 9D)**. 50 μM DMF significantly induced HMOX1 expression in human fetal **(Fig. 8E)** and adult **(Fig. 9E)** microglia. DMF but not MMF application trended to increase NQO1 and OSGIN1 expression in fetal microglia **(Fig. 8F and G)** but not in adult microglia **(Fig. 9F and G)**. Microglia appeared to exhibit normal motility while exposed to 50 μM DMF and MMF over six hours (data not shown) and were not susceptible to cell death by DMF or MMF *in vitro* as observed by PI and Calcein AM staining **(Fig. 7G to L)**.

Human microglia express HCAR2 at levels greater than human astrocytes.

Despite the lack of functional response to MMF as shown in **(Fig. 8 and 9)**, human microglia **(Fig. 10A)** express the putative high affinity MMF receptor HCAR2 (GP109A) as do astrocytes **(Figure 10B)**.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MMF 10μM
MMF 50μM
+ LPS

Figure 9

Figure 9: DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression, and *induces* HMOX1 expression in human adult microglia (p.44). Human adult microglia were pre-treated for one hour with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for six hours. **(A-C)** 50 μM DMF but not MMF pre-treatment inhibits LPS induced TNF **(A)**, IL-6 **(B)** and IL-10 **(C)** production in adult microglia. Protein levels are expressed as percent of LPS-only condition (dotted horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, error not shown (n=3); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA with Geisser-Greenhouse correction; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; n=8. **(D)** 50 μM DMF pre-treatment of human adult microglia inhibits LPS-induced miR-155 expression. **(E-G)** DMF induced up-regulation of HMOX1 **(E)**, but not OSGIN1 **(F)**, or NQO1 **(G)** in human adult microglia. Grey bars indicate mean RNA level versus untreated condition (dotted horizontal line). Repeated measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus each other; $\alpha = 0.05$; * n=7 except OSGIN1 n=3.

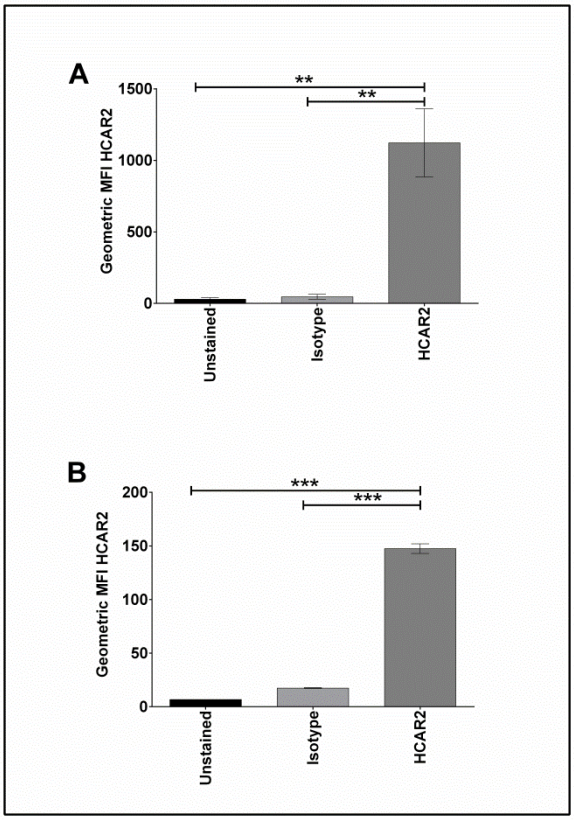

Figure 10: HCAR2 expression in human fetal microglia and astrocytes. Both human fetal microglia **(A)** and astrocytes **(B)** display specific HCAR2 staining by flow cytometry. Expression levels appear relatively higher in microglia. Repeated measures one-way ANOVA with Tukey's multiple comparisons test all columns versus each other; $\alpha = 0.05$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, etc.

Discussion

Our combined analysis of circulating monocytes from DMF-treated MS patients and of the *in vitro* responses of blood and brain-derived myeloid cells to DMF and MMF provides insights and challenges regarding defining the mechanism of action of this therapy. Our study focussed on both inflammation and oxidant related responses. Compared to controls, untreated RRMS patients had elevated levels of the anti-oxidant genes HMOX1 and OSGIN1 in monocytes. Previous reports described a decrease in HMOX1 in the total PBMC population of such MS patients versus healthy controls [226, 227] with a further decrease in both peripheral blood and CSF during relapse. We did not observe an increase in NQO1 expression, which indicates specificity of these genes in the response of myeloid cells to the MS disease process. We did not specifically examine other cell constituents comprising the PBMC population in our study. Based on reports that HMOX1 can serve an anti-inflammatory function [221-223], we speculate that its increased expression in MS patients reflects a response to the chronic inflammatory state and an attempt to provide negative feedback and down-regulate this activity.

The relative increase in circulating monocytes observed in our DMF-treated patients, accompanied by a reduction in lymphocytes is consistent with previous studies [228]. We found no evidence that such cells derived from DMF treated patients had a shorter life span *ex vivo* or were more sensitive to exposure to MMF or DMF. We observed a significant down-regulation of miR-155 and of HMOX1, and OSGIN1 expression in CD14+ monocytes of DMF treated patients compared to non-treated individuals. We also observed such results in patients treated with FTY720, although addition of FTY720 *in vitro* did not re-produce this effect (data not shown). Such results may indicate that the *in vivo* effects reflect an indirect response to overall reduction in inflammatory activity rather than a direct effect of the agent on these cellular pathways.

To gain more direct insights into the mechanism of action of DMF therapy, we added DMF or MMF to human monocytes *in vitro*. Over a wide concentration range, we observed no effect of MMF on cell survival or LPS induced cytokine production and anti-oxidant gene expression. This lack of effect of MMF contrasts with the report of

Asadullah et al., which found that MMF (100 μM) induced TNF and IL10 in human monocytes[218]. *In vitro,* MMF is also reported to enhance IL-4 and IL-5 production in total PBMCs and isolated primed T cells[229]. As mentioned, we did not specifically examine effects of MMF on other cellular constituents of PBMCs in our study. Consistent with our results, Lehman et al. also found that DMF, but not MMF, decreased inflammatory cytokine production in PBMCs[219]. However, our observation that DMF beginning at 10 μM induces cytotoxicity in monocytes makes it difficult to ascribe the observed reduction of LPS induced cytokine production and anti-oxidant gene expression to drug effect on a specific cellular signalling pathway.

In contrast to the results using human monocytes, our analysis of MDMs and microglia, indicated that we could observe effects of DMF on cytokine production and anti-oxidant gene expression in absence of overt cell cytotoxicity as observed by Calcein AM and PI staining, as well as live DIC imaging (not shown). Although the precise mechanism underlying this differential susceptibility cannot be defined, we note that in vivo microglia are long lived cells compared to monocytes. *In vitro* we found no measurable cell death of MDMs or microglia under basal conditions, whereas we noticed significant levels of death with monocytes at 24 hours (3.7 to 25.1%). The MDMs generated *in vitro* receive Macrophage Colony-Stimulating Factor (M-CSF) whereas monocytes do not. Microglia, unlike their rodent counterparts, will survive long term in culture even in absence of growth factor supplementation [1].

Over the dose range tested for both fetal and adult human microglia, we observed enhanced responses to DMF compared to MDMs. MDMs and microglia both up-regulated HMOX1 in response to 50 μM DMF. Again for both these cell types we did not observe functional effects of MMF although we could demonstrate presence of the HCAR2 receptor [212, 213]. For DMF, we observed inhibition of LPS-induced TNF, IL-6, and IL-10 in human microglia with 50 μM DMF pre-treatment *in vitro*. DMF also decreased mRNA for these cytokines, indicating a transcriptional level regulation. DMF has previously been reported to decrease TNF and IL-6 mRNA in rat microglia *in vitro*[220]. The observation that miR-155 was inhibited by DMF pre-treatment in microglia could indicate that inhibition of TNF occurs through multiple mechanisms including

transcriptional and post-transcriptional control. The observation that DMF upregulated HMOX1, but not OSGIN1 or NQO1 expression, in the adult microglia indicates a selectivity of the Nrf2 regulated response and/or involvement of other distinct regulatory pathways. The latter is further suggested by the lack of Nrf2 regulated anti-oxidant gene induction by MMF effects on the microglia. DMF induced glutathione depletion has been shown to induce transcription of HMOX1 [219, 221, 230-232].

With regard to the differential *in vitro* effects of DMF and MMF, fumarate treatment is recognized to induce transient oxidation of cells that in turn results in anti-oxidant gene expression. DMF and MMF both have a reactive pi (double) bond that confers this oxidant activity to the drugs. This double bond undergoes Michael-type addition reactions making it an effective glutathione depletion reagent, while oxidizing other thiols such as those that occur on appropriately exposed cysteine residues of proteins [204, 214, 233]. In this respect, DMF is shown to be significantly more potent than MMF [204, 230, 233, 234].

Conclusion

Our findings suggest that DMF therapy reduces systemic myeloid cell inflammatory and anti-oxidant gene expression *in vivo.* Compared to MMF, the lack of toxicity *in vitro* suggests that this may be an indirect effect reflecting overall reduction in disease activity, a finding supported by the observation that FTY720 therapy induces similar effects in MS patients. Our *in vitro* results using monocyte-derived macrophages and human brain-derived microglia suggest that fumarate therapy can induce a non-cytotoxic effect on cytokine and anti-oxidant gene expression on myeloid cells found within the CNS in MS. These effects were only mediated by DMF (not MMF), questioning the mechanisms whereby DMF therapy would modulate myeloid cell properties within the CNS.

PART 4: APPENDICES

Appendix 1: Cell Culture Methodology

Appendix 1A: Preparation of Pure Cultures of Human Microglia for *in vitro* experimentation [224]

Adult Tissue (work in sterile hood):

1. *Turn on shaking water bath (37° C). Pre-warm culture medium, DNAse and Trypsin aliquots.*
2. Tissue arrives in CUSA container or similar container. If tissue is dissociated into small pieces (less than $1mm^3$) proceed to step 3.
3. Mechanically dissociate tissue in a culture dish with sterile razors.
4. Pour tissue from CUSA container into 50 mL conical tubes (max 14).
    a. Let tissue settle out in conical tubes, pour off ¾ liquid, add more to tubes and repeat until CUSA container is empty.
    b. Rinse container with PBS to get all tissue into conical tubes.
5. Let tissue settle out and pour off ¾ liquid as above, but add PBS to wash. Repeat until liquid gets clear).
6. Pool all tissue to one tube.
    a. Prepare pathology cassette from 1 mL of tissue.
    b. Remove all blood clots using Pasteur pipette.
7. Pour cleaned tissue into a 100 mL bottle and rinse the conical tube with some PBS.
8. Add one aliquot DNAse and Trypsin for 15 mL of tissue, if more, add one more aliquot each. Top the bottle to 68 mL with PBS, cap and parafilm bottle.
9. Orbital water bath 30 mins, 180 RPM.

10. Add 10 mL FCS to stop trypsinization.

Filtration:

1. *Set up mashing filter and get a 20 mL syringe.*

- Do not exceed (50 mL x tube #) total volume during filtration!!
- 1-2 mL of tissue per tube

2. Pour some tissue into filter, mash, rinse with PBS, repeat until all the tissue is filtered. Rinse the 100 mL bottle with PBS well.
3. Divide the contents of the bottle into 50 mL conical tubes, top to 50 mL with PBS.
4. Spin at 300 x *g* (1350 RPM *on GH 3.8 Rotor*) for 10 min

Separation:

1. *During spin, prepare an equal number of sterile centrifuge tubes, each with 9 mL of percoll, and a 50 mL conical tube with ~25 mL PBS.*
2. After spin, decant supernatant and add <20 mL PBS to each tube. Re-suspend well.
3. Add 10 mL of tissue solution to centrifuge tube using pipette aid. Then take the rest and top pipette up to 11 mL using the PBS in the conical tube prepared in step 1. Transfer all tubes.
4. Weigh centrifuge tubes to match for balance. Centrifuge tolerance is 0.05 g (Add 10 uL PBS for each 0.01g difference).
5. Spin in ultracentrifuge on program 2- 31,000 x *g* (30,000 RPM), no brakes (45 minutes total time).

Isolation:

1. *Prepare a vacuum aspirator with some bleach and prep an equal number of 50 mL conical tubes.*

2. Remove the top layer of myelin using a vacuum aspirator
3. Transfer the opaque glial layer to the 50 mL conical tubes being careful not to disturb the RBC layer. Can plate the remaining fraction separately.
4. Top-up tubes to 50 mL with PBS, invert to mix.
5. Spin at 900 x *g* (2350 RPM *on GH 3.8 Rotor*) for 10 min, high brakes.
6. Decant supernatant as much as possible.
7. Add 7 mL media to one tube, re-suspend and transfer to the next tube. Repeat until all the pellets have been combined and re-suspended.
8. Use some media (<10 mL) to wash each tube by transferring as in step 7.
9. Spin at 300 x *g* (1350 RPM *on GH 3.8 Rotor*) for 10 min, low brakes.
10. Re-suspend in 2 mL media and remove 10 uL to count.
11. Plate at 2M x 10^6 cells/mL (growth density) in MEM with 5% heat-inactivated fetal calf serum (FCS), 0.1% Glucose, 1% Penicillin-Streptomycin (P/S), and 1% Glutamine.

Fetal Tissue (work in sterile hood):

1. *Turn on shaking water bath (37° C). Pre-warm culture medium, DNAse and Trypsin aliquots.*
2. Let tissue settle out and pour off ¾ liquid as above, but add PBS to wash. Repeat until liquid gets clear).
3. Decant ¾ liquid and pour contents onto petri dish. Remove meninges and blood clots if present using sterile forceps.
4. Use scalpels to cross-cut tissue into smaller pieces.
5. Add PBS and transfer to a 100 mL bottle using a pipette aid. Rinse the dish with some PBS. Top up to a final volume of 40 mL with PBS.

6. Add two aliquots DNAse and one aliquot Trypsin for 20 mL of tissue, if more, add one more aliquot each. Top the bottle to 68 mL with PBS, cap and parafilm bottle.

7. Orbital water bath 15 mins, 180 RPM.

8. Add 5 mL FCS to stop trypsinization.

Filtration:

2. Set up mashing filter and get a 20 mL syringe.

- Do not exceed (50 mL x tube #) total volume during filtration!!
- 1-2 mL of tissue per tube

3. Pour some tissue into filter, mash, rinse with PBS, repeat until all the tissue is filtered. Rinse the 100 mL bottle with PBS well.

5. Divide the contents of the bottle into 50 mL conical tubes, top to 50 mL with PBS.

6. Spin at 450 x *g* (1660 RPM *on GH 3.8 Rotor*) for 10 min

Separation and Isolation:

6. After spin, decant supernatant carefully but leave 10-15 mL liquid. Top up to 5 mL with media.

7. Transfer this first volume to the next tube to re-suspend and continue until all pellets have been re-suspended, and combined. Wash the empty tubes in a similar manner with some PBS.

8. Take 10 uL of the tissue to count (note the sample may need to be diluted 1:10 if the prep is large).

9. Spin at 300 x *g* (1350 RPM *on GH 3.8 Rotor*) for 10 minutes.

10. Pour off supernatant and re-suspend in 20 mL media. If the suspension contains clumps of dead cells it can be passed through a 70 um strainer.

11. Plate cells at $6x10^6$ cells/mL (40-50 mL in T175 or 15-20 mL in T75) in DMEM with 5% FCS, 0.1% Glucose, 1% P/S, and 1% Glutamine.

For both adult and fetal microglia cultures, media will need to be replaced after 5-7 days in culture. This is achieved by decanting spent media from flasks into 50 mL conical tubes, centrifuging at 1200 RPM for 10 mins, discarding spent media, resuspending pellet in new media, and adding this back to the original flasks which still contain a sub- or confluent astrocyte layer (fetal) or adherent microglia (adult). It is important to add a covering volume of PBS to the flasks and replace them at 37°C during the centrifugation step to ensure adherent cells remain healthy.

Appendix 1B: Isolation of Monocytes from Peripheral Blood Mononuclear Cells by Density Gradient Centrifugation and *in vitro* differentiation of Monocyte-Derived Macrophages

1. Obtain human blood in 8-12 heparinised vials (purple or green tubes).
2. Pour two vials (~20 mL) into one 50 mL falcon tube.
    a. Rinse the tube once with PBS.
3. Fill tubes with PBS to final volume of 35 mL.
4. Prepare spinal needle and 60 mL syringe- fill with Ficoll-Hypaque solution.
5. Place needle at the bottom of the falcon tube and *SLOWLY* plunge in 15 mL Ficoll.
6. Centrifuge 1800 rpm x 30 min, **BRAKES OFF** (45 min total time).
    a. 3 layers formed:
        i. CLEAR LAYER, PBS + SERUM
        ii. WHITE LAYER, PBMCs
        iii. RED LAYER, RBCs
7. Pipette off ~18 mL serum into waste basket using 10 mL falcon transfer pipette.
8. Pipette each layer of PBMCs into a separate new 50 mL falcon tube.
9. Fill tube to 50 mL with PBS and invert to mix well.
10. Centrifuge 1500 rpm x 15 min, **BRAKES ON**.
11. Decant supernatant, scratch pellet to resuspend, pool all cells to two tubes.
12. Add PBS to 50 mL, spin 1200 rpm x 10 min.
13. Decant supernatant, scratch pellet to resuspend, pool all cells to one tube.

14. Count cells, spin tubes at 1200 rpm x 10 min.
15. Resuspend cells in 80 uL Ice Cold MACS Buffer per 10 million cells and 20 uL Ice Cold MACS Beads per 10 million cells.
16. Incubate 15 min at 4 degrees C.
17. Pre-wet CD14 MACS columns with 3 mL MACS buffer (LS columns; 300 uL for MS column).
18. Resuspend pellets in 1 mL MACS buffer and add to column.
    a. Be precise in measurement of 1 mL to keep track of cell number.
19. Wait until all liquid has drained from column cistern, wash columns with MACS buffer (3 mL for LS; 300 uL for MS) 3 times.
20. Prepare a new, labeled falcon tube for the CD14+ fraction.
21. Add 5 mL MACS buffer to column remove the column from the magnet holder promptly and plunge the column to collect the CD14+ fraction.
22. Resuspend in 20 mL MACS buffer, count cells, spin at 1200 rpm x 10 min.
23. Resuspend in RPMI complete media (0.5x10^6 c/mL)
    a. Complete= 10% FCS, glutamine, P/S
24. Add M-CSF (1:400; stock 10 ug/mL) and replenish full amount via half media change after 3 days in culture.

Appendix 1C: Preparation of Pure Cultures of Human Fetal Astrocytes for *In Vitro* Experimentation

Isolation:

See Appendix 1A- Fetal Tissue (Page 47) and complete all steps as indicated.

At final step, plate 50 million cells in DMEM with 10% FCS, P/S and Glutamine in Poly (L) Lysine coated T75 vented tissue-culture flasks. Cells must be incubated at 37°C, 5% CO_2, for one week (or until confluent) before passaging.

Passaging and Purification:

When confluent, astrocytes should be split 1:2. Aspirate media from flasks, wash once in PBS, and incubate 5 minutes at 37°C, 5% CO_2, in 1X Trypsin and 2 mM ethylenediaminetetraacetic acid (EDTA). Cells should then be immediately placed in a 50 mL conical tube containing 5 mL FCS. Tubes should be topped up to 50 mL with PBS before centrifuging 10 minutes at 300 x *g.* Cells may then be split 1:2 in fresh media. Astrocytes can be used for experiments on their third passage.

Appendix 2: Assay Methodology

Appendix 2A: Methods for Quantification of RNA by Real-Time, Quantitative Polymerase Chain Reaction

RNA Extraction

Turn on centrifuge and set to 4°C before extraction, label clean, RNAse free 1.5mL eppendorf tubes.

1. Add chloroform at 1/5 the volume of trizol to each sample.
2. Spin at 16000 x *g* (13000 RPM), **4°C** for 15 minutes.
3. Label columns; warm up “RDD Buffer” (DNAse buffer) to 42°C and “Ultrapure H_2O” to 56°C using water baths.

NB: need about one RDD buffer tube (2 mL) for 24 samples

4. Following spin, pipette aqueous phase carefully into fresh tubes and keep track of volume.
5. Add 1:1 ice cold isopropanol to precipitate (can leave overnight at 4°C if necessary).
6. Add (max 700uL) isopropanol:sample mixture to labelled columns.
7. Spin at 16000 x *g* (13000 RPM), 4°C for 1 minute.
8. Discard flow-through in 50 mL conical tube.
9. For samples larger than 700uL, add remainder to same tube and repeat steps 7 & 8.
10. Add 350uL “RW1 Wash Buffer” to column.
11. Spin at 16000 x *g* (13000 RPM), 4°C for 1 minute
12. Discard flow-through in 50 mL conical tube.

Get DNAse from -20°C and warm RDD buffer. Will need (samples)/2 # aliquots.

13. Add 143 uL RDD buffer to each aliquot DNAse.
14. Add 80uL DNAse:RDD solution to each column. Incubate 15 min at 37°C.
15. Label a new set of clean, RNAse free 1.5mL eppendorf tubes.
16. Add 350uL RW1 wash buffer to columns.
17. Spin at 16000 x *g* (13000 RPM), 4°C for 1 minute.
18. Discard flow-through in 50 mL conical tube.
19. Add 500uL “RPE Wash Buffer” to samples (check that buffer has been reconstituted).
20. Spin at 16000 x *g* (13000 RPM), 4°C for 1 minute.
21. Discard flow-through in 50 mL conical tube.
22. Add 500uL 80% etOH (made with Ultrapure H_2O).
23. Spin at 16000 x *g* (13000 RPM), 4°C for 2 minutes.
24. Discard flow-through in 50 mL conical tube.
25. Spin dry; 16000 x *g* (13000 RPM), 4°C for 5 minutes.
26. Discard flow-through in 50 mL conical tube.
27. Elution in 20uL 56°C ultrapure H_2O; spin at 16000 x *g* (13000 RPM), 4°C for 1 minute.

Go to nanodrop™; bring 20uL tips, pipette, and samples and ultrapure water sample for blank.

28. Blank sample with ultrapure water.

29. Add 1.5uL of sample, enter sample name, read, wipe both contacts with kim wipe. Repeat.

30. Check 260/280 ratio to ensure it's over 1.5 for quality control.

Reverse Transcription for Gene Expression Quantitation

Before starting, RNA must be extracted from trizol.

1. Convert RNA concentration to sample volume required to run 100 ng of RNA.
2. Label a set of 500uL eppendorf tubes with sample numbers.
3. Prepare 100ng RNA samples in ultrapure water:
    a. Add (18uL-sample volume) ultrapure water to corresponding labelled eppendorf tubes.
    b. Add sample volume RNA to bring each sample to 18uL.

Master Mix for gene expression qPCR	
Ingredient	**Volume/ sample**
Random Hexaprimers (100uM)	3 uL
RT Buffer 5x	6 uL
dNTP (10mM)	1.5 uL
RNAse Out (40 u/uL)	1.33 uL
DTT	1 uL
MMLV-RT	2 uL

4. Multiply ingredients in this recipe by (sample # + 2) and make master mix.
5. Add 12 uL master mix to each tube.
6. Add samples to machine. Choose file 33, start.

qPCR Protocol

1. Make master mixes for each gene of interest including appropriate housekeeping gene.

   Master Mix Calculation (for duplicates):

   (Samples + NTC)*2.3*1.1 = multiplication factor *mf*

   A. Master Mix 2x: 5 uL * *mf*
   B. Ultrapure water: 4.2 uL * *mf*
   C. Probe: 0.8 uL * *mf*

2. Add 10 uL master mix to each well and 1 uL sample cDNA to appropriate wells.
3. Seal plate with sticker.
4. Spin to 1200 RPM and stop.
5. Place compression pad on top of sealed plate.

Go to qPCR machine. Bring cotton swab and alcohol to clean machine wells if necessary.

6. Turn on computer, open 7000 system software
7. Add plate to machine and close. Wait for machine to “click” and to see “connected” on 7000 software browser.
8. Add detectors, create new if necessary. Use FAM dyes only.
9. Add sample numbers to well inspector in program. Make sure to label reporters and NTCs correctly.
10. Instrument tab> 10uL (IMPORTANT)
11. File> Save As in folder of choice.
12. START (1:48 is run time)

Appendix 2B: Colorimetric Enzyme Linked Immunosorbance Assay (ELISA)

Plate/ Capture Prep

*This step should be one **1 day before** ELISA is planned.*

Set-up plates and mark them accordingly. Get ELISA kits from walk-in refrigerator and ELISA coating buffer (as per manufacturer specifications). Each plate requires that 12 mL capture antibody/coating buffer be mixed. Pour 12 mL coating buffer into a conical tube, add 48uL capture antibody (based on 1:250 recommended dilution). Invert to mix and add 100 uL capture/coating mixture to each well. Seal with plate stickers and store at 4°C overnight.

Standard and Working Solution Prep

*The following steps are done on **the day of** the ELISA.*

Materials:

A. Standards: lay out 8 eppendorf tubes for a linear serial dilution in 1:2 steps from a starting concentration according to manufacturer's specification.
    a. Samples must be diluted in ELISA diluent according to optimized protocols to a total volume of 100 uL. Currently, most samples can be detected within the standard range (defined according to manufacturer's specification) by using the following sample dilutions: IL- 1b (neat), IL-6 (1:20), IL-10 (1:2), IL-12 (1:2), TNFa (1:5).
    b. Cytokine standards should be kept at 100 ng/mL at -80°C.
B. Dilute 20 uL stock (100 ng/mL) in 980 uL assay diluent (1:50) for 2000 pg/mL standard solution, then dilute the rest 1:1.
C. To make working detector, mix 12 mL ELISA diluent with detection antibody (1:250) and enzyme reagent (SAv-HRP reagent; 1:250).
D. To make substrate solution, mix 12 mL (1:1) TMB and Hydrogen Peroxide. **NB. Make no earlier than 15 minutes before use.**
E. To make ELISA diluent, mix 500 mL bottle of PBS with 50 mL tube FCS (10%).

Capture/Detection

1. Aspirate/wash plates with excessive wash buffer and slam plates (careful!) to clear them (x2).
2. Block plates with 100 uL/ well ELISA diluent and leave sealed @**RT for 1 hour.**
3. Aspirate/wash plates as above (x2).
4. Pipette 100 uL of standard or sample** into each well, leave sealed @**RT for 2 hours.**
5. Aspirate/wash plates as above (x2).
6. Pipette 100 uL of working detector into each well; leave sealed @**RT for 1 hour.**
7. Aspirate/wash plates as above (x2).
8. Pipette 100 uL substrate solution into each well; leave sealed @**RT until you can see faint blue in lowest standard well.**
9. Pipette 50 uL H_2SO_4 (2.00 Normal) into each well to stop reaction.

Analysis

1. Turn on plate reader and carry out analysis according to software workflow.

Appendix 2C: Methods for Staining and Quantification of Cellular Protein by Flow Cytometry

Collect and Block:

1. Remove supernatants and use 37°C 2mM EDTA in PBS by swirling in wells and/or scraping gently to remove cells. Add cell mixture to 15 mL conical tubes pre-loaded with 2 mL serum.
2. Spin cells for 10 min at 234 x *g* and resuspend in 120 µL FACS buffer + 50 µL normal mouse Immunoglobulin G + 12.5 µL Normal Human Serum to block; incubate minimum 30 minutes at 4°C or overnight.

Staining:

1. Split cells in 96 well v-bottom plate.
    - When planning, account for unstained, single-stained, isotype-stained, and other control conditions (E.g. live/dead controls)
2. Spin 4 min at 416 x *g* and lightly vortex plate.

Go to step 10 for cocktail involving unconjugated antibodies or continue.

3. Resuspend in 50 µL antibody (Ab) master mix.
    - Unstained in FACS buffer alone, single-stained and isotypes in single Ab + FACS buffer to 50 µL total volume
4. Incubate 20-30 min in the dark on ice.
5. Add 100 uL FACS buffer to each well.
6. Spin 4 min at 416 x *g* and lightly vortex plate.
7. Resuspend each well in 150 uL FACS buffer.

8. Spin 4 min at 416 x *g* and lightly vortex plate.

9. Resuspend each well in 150 uL 1% Formaldehyde in PBS and transfer to FACS tube.

Unconjugated antibodies:

10. Resuspend in 50 µL unconjugated Ab master mix (unstained in FACS buffer, single-stained and isotype in single Ab + FACS buffer to 50 µL total volume, others in FACS buffer).

11. Incubate 20-30 min in the dark on ice.

12. Wash 2 times in 150 uL FACS buffer, each time spinning 4 min at 416 x *g* followed by light vortex-ing. NB. The first wash will be an addition of 100 µL of FACS buffer to each well.

13. Add secondary Ab diluted in FACS buffer to a total of 50 µL.

14. Incubate 30 min at room temperature in the dark.

15. Wash to times in 150 uL FACS buffer, each time spinning 4 min at 416 x *g* followed by light vortex-ing. NB. The first wash will be an addition of 100 µL of FACS buffer to each well.

16. Add conjugated Ab, SS and isotypes.

17. Incubate 20-30 min in the dark on ice.

18. Add 100 µL FACS buffer to each well.

19. Spin 4 min at 416 x *g* and lightly vortex plate.

20. Resuspend each well in 150 µL FACS buffer.

21. Spin 4 min at 416 x *g* and lightly vortex plate.

22. Resuspend each well in 150 µL 1% Formaldehyde in PBS and transfer to FACS tube. Skip this step for intracellular staining.

Go to flow cytometer to acquire events or continue for intracellular staining.

23. Resuspend each well in 100 µl PFA-saponin buffer for fixation-permeabilization.
24. Incubate 10 minutes at room temperature in the dark.
25. Wash twice in saponin buffer. After each, spin 4 minutes at 416 x *g* and lightly vortex plate.
26. Resuspend cells in Ab master mix in saponin buffer to a final volume of 50 µl.

    - Unstained in saponin buffer alone, single-stained and isotypes in single Ab + saponin buffer to 50 µL total volume

27. Incubate 30 minutes in the dark on ice.
28. Wash twice in saponin buffer. After each, spin 4 minutes at 416 x *g* and lightly vortex plate.
29. Resuspend cells in 200 µl FACS buffer and transfer to FACS tubes.

Keep samples in the dark and go to flow cytometer for acquisition.

Appendix 2D: Methods for Immunofluorescence Staining and Imaging

Standard Immunostaining

Solutions and Reagents

1. Phosphate buffered saline (PBS), adjust pH to 8.0.
2. Paraformaldehyde (4% in PBS)
3. Blocking Buffer: 5% normal serum and 0.3% Triton X-100 in PBS
4. Antibody Dilution Buffer: 1% *w/v* Bovine Serum Albumin and 0.3% Triton X-100 in PBS.
5. Antibodies (primary, secondary, conjugated, etc)

Fixation

1. Aspirate culture supernatants and fix cells in fresh 4% PFA for 15 minutes at room temperature.
2. Wash three times in PBS (pH 8.0).

Staining

1. Block for 60 minutes in blocking buffer.
2. Aspirate blocking buffer and apply diluted primary or conjugated antibody in antibody buffer.
3. Incubate overnight at 4°C.
4. Wash three times in PBS (pH 8.0)
5. Incubate in diluted secondary antibody in antibody buffer for 1-2 hours at room temperature in the dark.
6. Wash three times in PBS (pH 8.0)
7. Coveslip slides or keep wells in PBS at 4°C until imaging. Protect from light.

Live/Dead Fluorescence Imaging

Cells will be imaged "live" in wells in PBS, immediately following staining with Calcein AM and Propidium Iodide.

1. Following experimental manipulation, aspirate (and/or collect) cell supernatants and wash cells gently in PBS twice.
2. Incubate cells with 4 µM Calcein AM and 1 µM Propidium Iodide for 20 minutes at 37 °C with 5% CO_2. Remember to include a positive control for cell death in one well by incubating in the presence of 0.1% Saponin or similar (e.g. Triton-X 100).
3. Wash cells twice in PBS and image immediately using a standard fluorescence microscope with green and red channels.
    a. PI emission/excitation = 493/636
    b. Calcein AM emission/excitation = 495/516

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