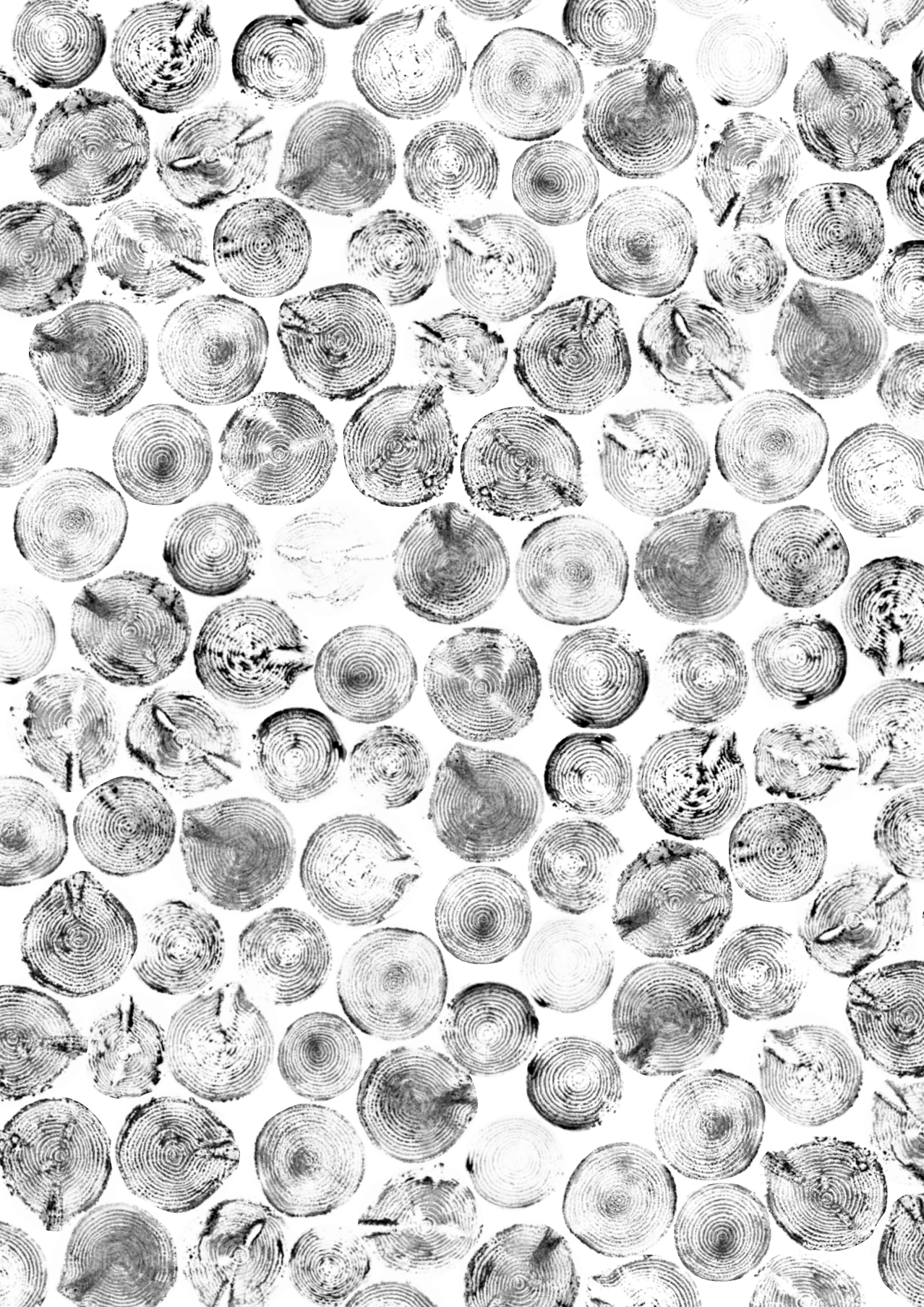
Thesis for doctoral degree (Ph.D.)
2020

Demyelinating conditions regulated by ROS pathways outside and inside the brain

Karl Carlström

Karolinska Institutet



From Department of Clinical Neuroscience
Karolinska Institutet, Stockholm, Sweden

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Stockholm 2020

Published by Karolinska Institutet.
Printed by Arkitektkopia AB, 2020

ISBN 978-91-7831-689-2

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By

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AKADEMISK AVHANDLING

Som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen kommer försvaras i CMM Lecture Hall, CMM L8:04, Karolinska Universitetssjukhuset Solna, 17176 Stockholm.

Fredagen den 31 januari 2020 kl 09.00

Principal Supervisor:

Prof. Fredrik Piehl
Karolinska Institute
Department of Clinical Neuroscience
Neuroimmunology Unit

Co-supervisor(s):

Dr. Faiez Al Nimer
Karolinska Institute
Department of Clinical Neuroscience
Neuroimmunology Unit

Dr. Xingmei Zhang
Karolinska Institute
Department of Clinical Neuroscience
Neuroimmunology Unit

Prof. Staffan Holmin
Karolinska Institute
Department of Clinical Neuroscience
Neuro

Opponent:

Prof. Catherine Lubetzki
Hôpital Pitié Salpêtrière
Brain and Spine Institute

Examination Board:

Prof. Emeritus Ralf Morgenstern
Karolinska Institute
Institute of Environmental Medicine

Prof. Jonas Frisén
Karolinska Institute
Department of Cell and
Molecular Biology

Prof. Emeritus Jan Ernerudh
Linköping University
Department of Clinical and
Experimental Medicine

Therese

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory condition of the central nervous system with immune-mediated damage on myelinated nerve tracts. Accumulating evidence support a role for autoreactive T-lymphocytes orchestrating immune-attacks on myelinating oligodendrocytes (OLs). While oligodendrocyte precursor cells (OPCs) may initially compensate for loss of myelin by differentiating into OLs, later stages of MS are characterized by arrest of OPC differentiation and clinical disease progression. Reactive oxygen species (ROS) are important in many contexts, but their role in regulation of adaptive immune processes and remyelination in MS is still uncertain.

We here first characterized the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-activating MS-therapy dimethyl fumarate (DMF/ Tecfidera™) in cell cultures and an experimental model for neuroinflammation. In MS patients we assessed the effect of DMF on transcription and epigenetic modification in immune cells, as well as ROS production by monocytes. Our findings indicate that monocyte-derived ROS is linked to the clinical efficacy of DMF by modulating the adaptive immune response, in turn highlighting modulation of redox processes as a therapeutic target in autoimmune disease.

In order to study the effect of DMF and other redox active agents, we developed a cell based reporter system (pTRAF) that enabled the visualization of transcription factor activity in real-time. Using pTRAF and other *in vitro* and *in vivo* models we further characterized the downstream effects of DMF and a set of more selective Nrf2-activating compounds. Finally, we performed extensive *in vitro* and *in vivo* characterization of Glutathione S-transferase 4α (Gsta4), which is regulated by Nrf2 and serves an important role for scavenging of the lipid peroxidation product 4-hydroxynonenal (4-HNE). Interestingly, higher levels of Gsta4 was associated with faster and more complete remyelination by facilitating the differentiation of OPC into mature myelinating OLs. This was suggested to be mediated through modulation of the Fas/Casp8/Bid-axis, leading to increased survival of differentiating OPCs.

Collectively, our findings support a role for redox processes in regulating adaptive immune responses in MS. Furthermore, a specific redox-related process involving 4-HNE and Gsta4 was found to regulate OPC survival during remyelination. Both these mechanisms may represent interesting novel therapeutic targets in MS, as well as in other autoimmune and demyelinating conditions.

LIST OF SCIENTIFIC PAPERS

I. **Carlstrom K E**, Ewing E, Granqvist M, Gyllenberg A, Aeinehband S, Lind Enoksson S, Checa A, Badam T, Huang J, Gomez-Cabrero, Gustafsson M, Al Nimer F, Wheelock C, Kockum I, Olsson T, Jagodic M and Piehl F. *Therapeutic efficacy of dimethyl fumarate in relapsing-remitting multiple sclerosis associates with ROS pathway in monocytes.* **Nature Communications** 10, 3081 **(2019)**

II. Johansson K, Cebula M, Rengby O, Dreij K, **Carlstrom K E**, Sigmundur K, Piehl F and Arnér E. *Cross-talk in HEK293 cells between Nrf2, HIF, and NF-κB activities upon challenges with redox therapeutics characterized with single-cell resolution*. **Antioxidants & Redox Signaling**. 26, 229–246 **(2017)**.

III. **Carlstrom K E**, Chinthakindi P, Espinosa B, Al Nimer F, Arnér E, Arvidsson P, Piehl F and Johansson K. *Novel vinyl sulfone compounds are more specific Nrf2-activators in vitro and in the central nervous system than dimethyl fumarate.* **Submitted manuscript.**

IV. **Carlstrom K E**, Zhu K, Ewing E, Krabbendam I, Harris R, Mendanha Falcão A, Jagodic M, Castelo-Branco G and Piehl F. *Glutathione S-transferase 4α is a key regulator of oligodendrocyte differentiation and remyelination in demyelinating models of multiple sclerosis, by reducing caspase-8 activity and maintaining mitochondria integrity.* **Reviewed manuscript.**

LIST OF SCIENTIFIC PAPERS NOT IN THE THESIS

Dominguez C, **Carlstrom K E**, Zhang X, Al Nimer F, Lindblom R, Ortlieb Guerreiro-Cacais A and Piehl F. *Variability in C-type lectin receptors regulates neuropathic pain-like behavior after peripheral nerve injury*. **Molecular Pain**. 10, 78 **(2014)**.

Ineichen BV, Zhu K and **Carlström K E**. *Axonal mitochondria across species adjust in diameter depending on thickness of surrounding myelin.* **BioRxiv** (pre-print)

CONTENTS

ABBREVIATIONS

[-SH]	thiol goup
[$^{\bullet}$OH]	hydroxyl radical
[4-HNE]	4-hydroxynonenal/4-hydroxy-2-nonenal
[H_2O_2]	hydrogen peroxide
[LOO$^{\bullet}$]	lipid peroxyl radical
[$O_2^{\bullet-}$]	superoxide
[O_2]	(di)oxygen
APC	antigen presenting cell
ARE	anti-oxidative response element
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
Bid	BH3 interacting-domain death agonist
CC	corpus callosum
CCR2	C-C chemokine receptor 2
CF	clemastine fumarate
CNS	central nervous system
DA	Dark agouti
DMF	dimethyl fumarate
DMT	disease modulatory therapy
EAE	experimental autoimmune encephalomyelitis
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase regulatory subunit
GFP	green fluorescent protein
GLUT1	glucose transporter 1
HEK293	human embryonic kidney 293 cells
HIF	hypoxia inducible factor
IFA	incomplete Freud’s adjuvant
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor 1
Keap1	Kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
LPC	lysolechitin (lysophosphatidylcholine)
MBP	myelin basic protein

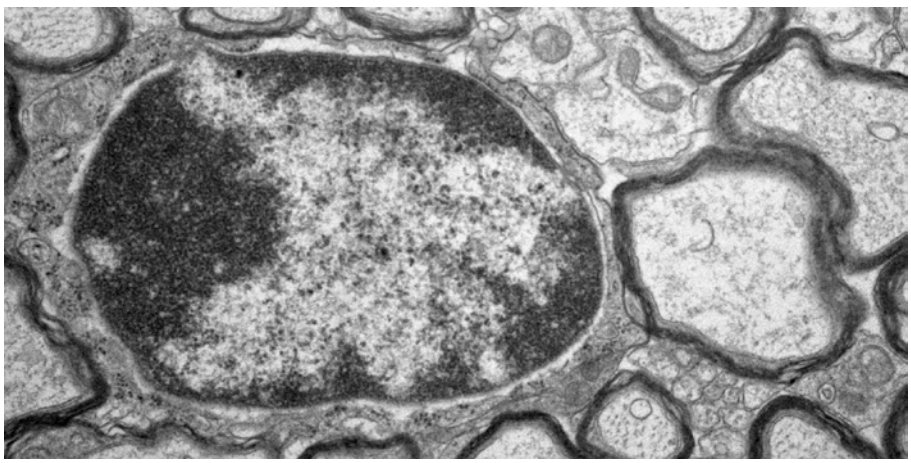
MCT	monocarboxylate transporter
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
NOX	(NADPH oxidase) nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OL	oligodendrocyte
pre-OL	premature oligodendrocyte
OPC	oligodendrocyte precursor cell
aOPC	adult oligodendrocyte precursor cell
PDGF	platelet-derived growth factor
PLP	myelin proteolipid protein
PPMS	primary progressive multipole sclerosis
pTRAF	plasmids for transcription factor reporter activation-based upon fluorescence
PUFA	polyunsaturated fatty acids
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RRMS	relapsing remitting Multiple multiple sclerosis
SNP	single nucleotide polymorphism
SPMS	secondary progressive Multiple multiple sclerosis
TBI	(experimental) traumatic brain injury
TCA	tricarboxylic acid cycle
T_H1	type 1 helper T cell
T_H17	type 17 helper T cell
TNF	tumor necrosis factor
T_{reg}	regulatory T cell

1 INTRODUCTION

1.1 Prologue

Myelin associated to nerve fibers was first described by Antonie van Leeuwenhoek (b.1632) using his latest invention, the microscope[1]. In 1781 Felice Gaspar Fontana (b.1730) further described the large number of uniform cylinders surrounding the nerve as “basic organic elements of nerves”. Almost 150 years later Pío del Río-Hortega (b.1882) named them oligodendroglia and suggested that it to be the cell type producing myelin. Betty Geren (b.1922) together with Mary Bunge (b.1941) and Richard Bunge (b.1932), all pioneers in electron microscopy, confirmed Hortega’s observation with their caption of myelin sheaths from an oligodendrocyte wrapped around a transected axon. This discovery was made nearly three centuries after van Leeuwenhoek´s initial discovery using his much simpler microscope.

In the image, you can see a phagocytic cell maybe interacting with a myelinated and transected axon.



1.2 Redox regulation

Reactive oxygen species (ROS) are small chemically reactive oxygen-derived molecules that may be derived from multiple sources, including: mitochondria, NADPH oxidases, lipoxygenases and peroxidases.

Oxidative stress is the term for incapacity to sufficiently scavenge ROS in a biological system. Unless scavenged ROS avidly reacts chemically and non-specifically with surroundings biomolecules, such as nucleic acids, carbohydrates, lipids and proteins, which may cause irreversible damage and loss of function. During the interaction between ROS and surrounding biomolecules of the cell, electrons (e^-) are transferred from donor to acceptor compound. Compounds that donate e^- are reductants and compounds that take up e^- are oxidants. Fundamental physics state that reductants and oxidants always coexist since an e^- cannot be generated or destroyed; a coexistence that can be referred to as *redox*.

Multiple transcription factors are engaged during redox regulation in health and disease[2–5].

The primary defense against oxidative stress is orchestrated in a delicate fashion by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is kept in the cytoplasm by the oxidative stress sensitive Kelch-like ECH-associated protein 1 (Keap1) in complex with Cul3 E3 ligase[6] (Fig.1). In the absence of oxidative stress Keap1 captures Nrf2 in the cytosol, where it is continuously ubiquitinated and subsequently degraded in the proteasome[7]. In the presence of oxidative stress, the thiol-containing (-SH) Keap1 is oxidized, undergoes conformational change and dissociates from Nrf2[8,9]. Nrf2 will then be able to translocate to the cell nucleus and dimerize with different members of the small Maf protein family and bind to anti-oxidative response elements (ARE)[8,9]. The Nrf2-Maf heterodimers are able to regulate the expression of about 200 transcripts[6,7,10–12]. These transcripts largely belong to two pathways: the thioredoxin and the glutathione system, together essential to maintain redox homeostasis and to prevent oxidative stress[13–15]. Key-players in the thioredoxin system are thioredoxin and thioredoxin reductase. Glutamate-cysteine ligase regulatory subunit (GCLM) and glutamate-cysteine ligase catalytic subunit (GCLC) generate one of the main antioxidants, namely the non-protein thiol glutathione (GSH) and accordingly belongs to the glutathione system. Both systems are overlapping, since knock-out experiments have illustrated that both pathways adapt to compensate for the loss of the other[16]. However, disruption of both pathways is associated with a lethal phenotype[17].

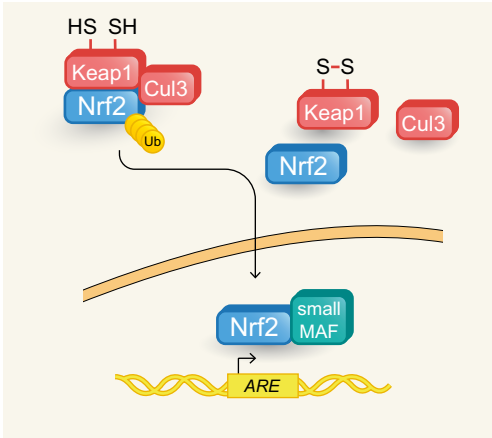


Figure 1. *Illustration of Nrf2 signaling pathway.*

1.21 Mitochondrial ROS in the brain

Mitochondria play an essential role in supplying eukaryotic cells with energy via adenosine triphosphate (ATP) generated from oxygen (O_2). In addition, mitochondria serve important roles for Ca^{2+}-buffering, iron storage and apoptosis[18,19]. ROS generated from mitochondria (also referred to as mROS) can be regarded as a by-product of redox processes, where it is estimated that during the conversion of O_2 to ATP 2–5% is instead converted into ROS. This is sometimes referred to as *leaky mitochondria*[20]. The generation of ATP from oxygen in the mitochondria is essential for cells and thus requires extensive control to be maintained. In addition, due to the reactive nature of ROS intermediates, all processes must be tightly controlled to avoid by-stander damage induced by ROS and cellular elements. Despite mitochondria being a ubiquitous organelle, symptoms from primary mitochondria disorders are often related to the central nervous system (CNS), likely due to the high energy demand[21]. Thus, if focusing on mitochondria in the brain, Na^+/K^+ ATPase facilitates the transmembrane transport of sodium (export) and potassium (import) along the axons to maintain axonal resting potential. During homeostasis, 70% of the axonal mitochondria are located close to the node of Ranvier, where the Na^+/K^+ ATPases are present in myelinated axons[21,22]. Rapid shifts in cellular potential occurring during axonal signal conduction make Na^+/K^+ ATPases a major energy consumer in the CNS[23].

Myelination of larger axons not only facilitates proper signal transduction but also favor efficient use of energy (reviewed in section 1.41).

During homeostasis there is a variance in axonal mitochondria density between different CNS tracts that correlates with the level of myelination and axonal diameter[24,25]. Furthermore, several research groups have described an increase and redistribution of energy-dependent ion-channels in response to insufficient axonal insulation[26,27]. The loss of proper axonal insulation would thus be linked to increased energy demand. This notion is also supported by findings in the Shiverer mouse, which lacks the ability to produce sufficient myelin due to a mutation in the gene coding for myelin basic protein (MBP). Hence, the Shiverer mouse displays an increased mitochondrial density and higher activity of the Complex IV[28]. Similar results have been obtained in transgenic models targeting myelin proteolipid protein (PLP)[29]. In addition, an experimental model for demyelination using Theiler virus also displays higher mitochondria density in demyelinated compare to myelinated axons[30]. In line, mitochondrial dysfunction has been implicated also in a mouse model of Multiple sclerosis (MS), namely experimental autoimmune encephalomyelitis (EAE)[31], where the application of ROS scavengers prevented neurodegeneration[32–34]. Taken together these findings suggest that cells of the CNS are particularly sensitive to mitochondrial dysfunction.

1.22 Generation of ROS from NOX enzymes in the periphery

Perhaps due to the early discoveries of certain ROS scavengers, such as sodium dismutase (SOD), ROS often were regarded as something unwanted and potentially toxic during homeostasis[35]. Over the years this view has been revised to accommodate the characterization of dedicated producers of ROS, such as membrane-associated nicotinamide adenine dinucleotide phosphate oxidase (NOX/NADPH oxidase) that transport NADPH-derived e^- across the membrane to reduce oxygen, O_2 to superoxide, $[O_2^{\bullet-}]$ [18,36]. In addition, NOX enzymes are expressed by a diversity of cells during homeostasis, thus further challenging the view that generation of ROS is restricted to pathological conditions[37].

The NOX family consists of seven members (NOX1-5 and DOUX1-2) where NOX1-4 all share the subunit $gp22^{phox}$ but otherwise are made up by different subunits.

The importance of NOX was identified in patients with chronic granulomatous disease. These patients showed increased susceptibility for bacterial infection despite that their leukocytes maintained functional phagocytic and chemotactic functions. However their phagocytes were not able to burst generating superoxide, $[O_2^{\bullet-}]$ and hydrogen peroxide (H_2O_2) to break bacterial DNA[38,39]. To further verify the role of NOX in innate immunity, deletion of NOX2 or $gp22^{phox}$ gave rise to a similar phenotype in mice[38].

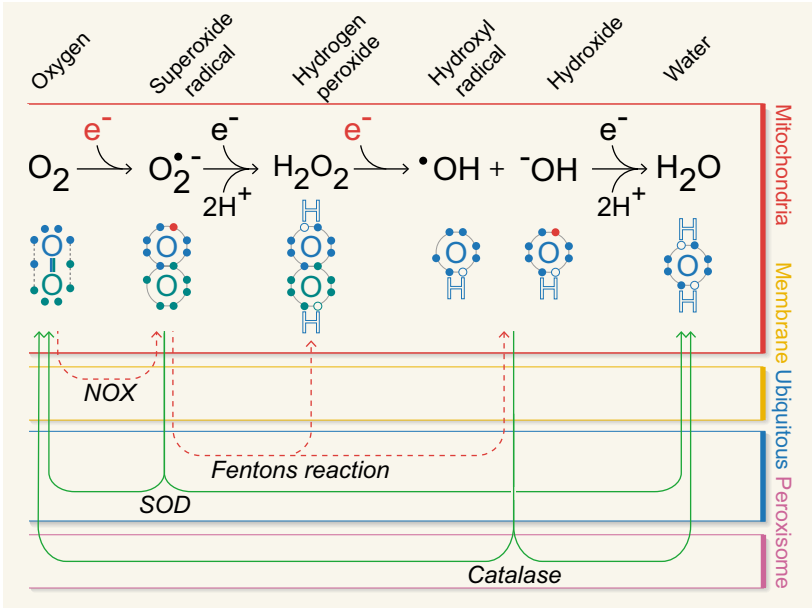


Figure 2. Illustration of all ROS intermediates. *Colored labels to the right indicate subcellular location of the chemical reaction.*

1.23 Downstream effects of ROS

ROS modulate multiple intracellular signaling pathways and cell functions both in the cytosol and on the cellular surface, and this has been extensively reviewed[40,41]. One example of intracellular events influenced by ROS is the interplay between Nrf2 and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB)[42,43]. Another example is the oxidation of polyunsaturated fatty acids (PUFA) in the bilayer cell membrane that faces both the cytosol and the extracellular compartment by the ROS intermediate hydroxyl radical, [•OH]. Due to its high capability to be reduced, hydroxyl radical may initiate a rapid chain-like reaction with PUFA in the cell membrane, where the PUFAs forms lipid peroxyl radicals, [LOO•]. These radicals will further oxidize surrounding PUFAs of the cell membrane. This process is referred to as lipid peroxidation (LPO) and can rapidly cause destruction of PUFA-rich areas, such as the cell membrane. In this regard, myelin sheaths are also particularly sensitive due to their high density of lipids. When reduced, lipid peroxyl radicals release reactive aldehydes such as 4-hydroxynonenal (4-HNE), malondialdehyde and acrolein[44,45]. These reactive aldehydes are less reactive compared to ROS intermediates. To make an comparison with a ROS intermediate, hydroxyl radical has an estimated *in vivo* a half-life of 10^{-9} seconds[46], whereas the half-life of 4-HNE is up to two minutes[44]. This implies that 4-HNE can diffuse and

interact with biomolecules further away from the production origin compared to ROS intermediates. Just as ROS, 4-HNE may interfere with multiple homeostatic processes[47] and may bind DNA to cause proteasomal and mitochondrial dysfunction[48]. However, 4-HNE is restricted to form protein adducts via covalent interactions only with cysteine, histidine and lysine[44,49], which in turn makes certain protein structures more sensitive than others.

1.3 Monocyte development and subsets

Monocytes derive from granulocyte-macrophage progenitors in the bone marrow, a type of hematopoietic stem cell that also generates neutrophils, eosinophils, basophils and dendritic cells. During homeostasis, bone marrow-derived monocytes accounts for ~5–10% of all leukocytes in the blood[50]. The monocyte population both in human and mouse is heterogeneous and can be further classified with regard to nuclear morphology and size.

Human monocytes subsets in the circulation are mainly divided into two groups based on the expression of CD14, a part of the lipopolysaccharide (LPS) receptor, and CD16, the type III Fc-receptor γ (FcγRIII)[51]. A subset of monocytes, referred to as inflammatory monocytes ($CD14^{hi}CD16^{-}$) in addition express the C-C-chemokine receptor 2 (CCR2). They also displays phagocytic capacity and are able to produce significant amounts of cytokines, including tumor-necrosis factor (TNF) and interleukin-6[50]. Another subset is referred to as resident monocytes ($CD14^{+}CD16^{+}$) and displays high surface expression of MHC class II molecules and FcγRII[52]. Greismann *et al.* identified the mouse Ly6C epitope to overlap with the human $CCR2^{+}$ monocyte subset[53,54]. Based on this finding the mouse model have been the primary model to study functions of different monocyte subsets[53,54]. Thus, in mice inflammatory monocytes are identified by $Ly6C^{+}CCR2^{+}CX_3CR1^{+}$. Adoptive transfer of inflammatory monocytes expressing green fluorescent protein (GFP) have indicated these cells to have a lifespan of 20 hours during homeostasis[55]. In inflammatory conditions these $CCR2^{+}$ monocytes migrate to the tissue where they upregulate CD11c and MHC class II[50]. Later they will also acquire T cell priming capacities and become antigen presenting cells (APCs)[52]. CCR2 is also crucial for the process of exiting from the bone marrow and thus Ly6C is considered to be primarily be expressed on newly formed monocytes in the circulation[54], while $Ly6C^{-}CCR2^{-}CX_3CR1^{hi}$ monocytes are regarded as terminally differentiated[56].

In addition to $Ly6C^{+}$ monocytes, recent studies have also suggested an intermediate Ly6C monocyte population to represent a further differentiated form of APCs[57]. This suggests that inflammatory $Ly6C^{+}$ gives rise to macrophages with an iNOS signature whereas $Ly6C^{int}$ give rise to monocyte-derived dendritic cells.

1.31 NOX, ROS and CNS autoimmunity

The role of NOX in innate immune cells in infections is well described elsewhere and the focus in this section will thus focus on NOX-derived ROS in immune cell-regulation and its potential role in conditions of CNS autoimmunity.

EAE (described in detailed in section 2.5) is the commonly used experimental model for MS. The EAE model reproduces important hallmark events seen in the early relapsing-remitting phase of MS (RRMS), including priming of T cells in lymphoid tissues, CNS-infiltration of encephalitogenic T cells and the appearance of demyelinated lesions in the CNS. Disease activity in the EAE model is primarily driven by T cells, which is illustrated by the fact that adoptive transfer of encephalitogenic T cells is sufficient to elicit clinical disease[58]. In terms of numbers however, monocyte-derived cells are the most prominent CNS-infiltrating cells at the primary bout of disease[59,60]. The relevance of monocytes is underscored by the observation that rodents lacking circulating monocytes do not develop clinical EAE. Histological assessment of CNS-lesions also reveals the presence of NOX enzymes and ROS-mediated damage, suggesting also an important role for ROS pathways. Interestingly, generation of ROS from monocytes/monocyte-derived macrophages suppresses various T cell function resulting in anti-inflammatory outcomes[61–65] (Fig.3).

The role of monocytes in neuroinflammation is however complex, since the effects of circulating monocytes compared to monocytes infiltrating the tissue differ considerably. Thus depletion of superoxide [$O_2^{\bullet -}$] production due to knock-out of *NOX* causes break of T cell tolerance[66] and induction of autoimmune responses. Also loss of *NOX* further worsen autoimmune conditions[39,67–69]. In line with this, lack of ROS generation in monocytes/macrophages also increases T cell proliferation[70] and during antigen presentation alter lineage commitment to generate more T_H1, T_H17[71] and to lesser extent beneficial T_{reg}[72,73] (Fig.3). Interestingly, there are also supporting findings for a ROS-induced apoptotic-gradient among T cells subtypes, where the state of maturation and location appear to affect the level of susceptibility to ROS-induced apoptosis. Hence, naïve T cells in the thymus are sensitive[74], while circulating naïve T cells are not as sensitive as activated T cells[75,76]. Further, the induction of lymphocyte apoptosis depend on the intracellular depletion of anti-oxidative GSH where the presence of GSH prevent development of autoimmune responses[66,77,78].

Several studies have been conducted to pin-point endogenous T cell mechanisms being targeted by ROS. One approach to quantify the level of oxidation has been made via assessment of chemically reduced thiols [-SH] on the cell surface. An additional approach has been to assess the abundance of intracellular GSH during T cell activation using CD45RA, which is highly expressed on naïve T cells but

downregulated upon antigen-encounter. High levels of chemically reduced cell surface [-SH] on T cells arise due to low levels of oxidative ROS[78,79]. Further, chemically reduced cell surface [-SH] levels are diminished on T cells following clonal expansion and proliferation[79]. Interestingly, T cell cultured with reducing agents, thus increasing the presence of cell surface [-SH], attenuate the their responsiveness and proliferation[80].

The intracellular balance in redox state also have implications on transcriptional activities decisive for cell phenotype and/or lineage commitment. The transcription factors NFκB and Fork-head transcription factors of class O are both involved T cell homeostasis, maturation and survival and are both influenced by the intracellular redox state[81–83].

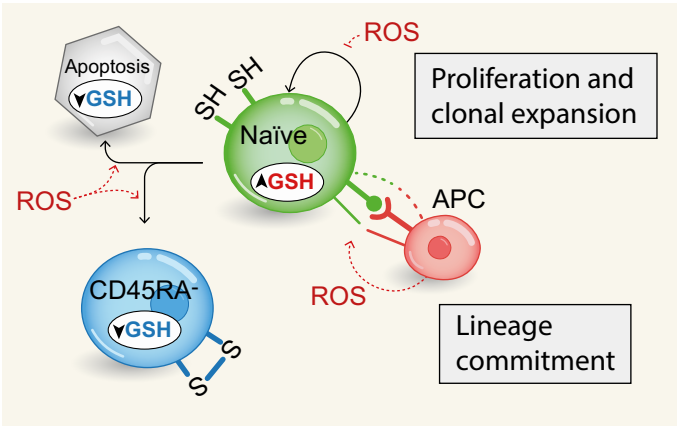


Figure 3. *Illustration of ROS-mediated alternations in immune responses and immune cell-cell interactions.*

1.32 Multiple sclerosis and dimethyl fumarate

Among pathological conditions with demyelinating components, MS is the most well-studied. MS represents a heterogeneous, demyelinating and neurodegenerative condition, with genetic associations to a large number of immune-related genes, in particular genes related to T cell functions[84–86]. In addition there are also environmental factors contributing to disease onset[87,88]. MS onset often occurs during the third or fourth decades of life and women are more than twice as susceptible as men[89]. Of the patients diagnosed with MS, about 80% initially present with a relapsing remitting disease course referred to as RRMS. These patients display bouts of neurological symptoms, which correlates with the infiltration of immune cells into the CNS[90]. Diagnostic hallmarks for MS and disease progression thus includes magnetic resonance imaging (MRI) visualizing plaques consisting of these infiltrating immune cells[90,91]. Despite large inter-individual differences, a

majority of patients with time will convert into a phase of unrelenting worsening; secondary progressive MS (SPMS). A minority of patients displays a progressive phenotype from start; primary progressive MS (PPMS)[92]. Progressive phases of MS are characterized by a steady decline in cognitive and neurological function largely independent of infiltrations of immune cells and the relapses seen in RRMS[85,93].

During homeostasis, $CD4^+$ T cells are present and patrolling the CNS. In disease however, the infiltrating $CD4^+$ T cells are reactivated by local APCs in the perivascular space, which trigger them to progress into the CNS parenchyma and produce cytokines such as TNF and interferon-γ (IFN-γ)[94]. These cytokines activate surrounding microglia and perivascular macrophages causing degradation of the myelin sheaths. Current disease modulatory therapies (DMTs) for MS mainly primary target the dysfunctional adaptive immune phenotype of MS. In particular, DMTs that restrict the recruitment of peripheral immune cells of the adaptive immune system to the CNS have been proven successful[89]. Examples include; S1PR antagonists inhibiting lymphocyte exit from the lymph nodes (fingolimod and more selective S1PRs), blocking of VLA-4 expressed on immune cells that prevents migration across the BBB (natalizumab) and DMTs that deplete specific immune cell populations (monoclonal anti-CD20 and anti-CD52, and cladribine) [89,95].

In contrast to these DMTs, the mechanism of action of dimethyl fumarate (DMF/ Tecfidera™) in MS, has been less clear. DMF is a first-line DMT for RRMS used in clinical routine in Sweden from from May 2014[96–98]. It was originally found to be effective for psoriasis and was discovered by serendipity to also improve clinical MS symptoms in a small number of patients suffering from both conditions[99]. Following this discovery, DMF was also shown to ameliorate clinical symptoms in EAE[100,101]. Among the suggested pathways targeted by DMF is the Hydroxycarboxylic acid receptor 2 in microglia[102]. Further, pre-clinical studies ascribed DMF a cytoprotective effect when used *in vitro* via the transcription factor Nrf2[103–106]. More recent studies verified the involvement of Nrf2[107], but also indicated peripheral immune cells as an important target[108–113]. It should also be noted that DMF is not specific towards Nrf2[114,115], and in fact may also affect immune cells independently of Nrf2[116,117].

1.4 Juvenile and adult oligodendrocyte development

During development, the generation of oligodendrocyte precursor cells (OPC) and their subsequent differentiation to mature myelinating cells of the CNS is tightly regulated[118–123]. During embryonic development, three waves of OPCs propagate from the forebrain, progressing in a dorsal-ventral manner[124,125]. Studies applying cell fate-mapping have shown a first wave of NK2 Homeobox 1 (Nkx2.1), Nkx2.2 and oligodendrocyte transcription factor 2 (Olig2) positive OPCs to populate the whole developing telencephalon, this is initiated at embryonic day 12.5 (E12.5)[126].

Nkx2.1 positive OPCs are to a large extent replaced by a second wave of Gsx2 positive OPCs initiated at E15.5, the latter wave will also persist postnatally[126,127]. At postnatal day 0 (P0), a third wave of OPC expressing Emx1 will progress in a dorsal-ventral pattern from the dorsal ventricular zone and first facilitate myelination in the deep cortical layers and then the superficial layers[128,129]. This dorsal-ventral pattern is also preserved during remyelination[129]. These OPCs generated during development have been described to be more motile and have a more rapid cell-cycle compared to adult OPCs (aOPC) which are generated during adult life[130,131]. This is also the reason for the common use of OPC from neonatal rodents to establish primary OPC cultures[132].

Nkx2.2 and Olig2 may regulate each other and their co-existence is essential to enable development into oligodendrocytes (OL). Olig2 directly induces SRY-box 10 (SOX10), a hallmark transcription factor that is essential for OL maturation and also exerts a positive feedback on Olig2. SOX10 interacts with several additional proteins to mediate OL differentiation. To date four different SOX10-mediated pathways of differentiation has been identified: (1) Myelin regulatory factor (MYRF), where SOX10 and MYRF show overlapping binding to transcriptional control regions close to transcripts involved in myelination; (2) Chromatin modulation via CHD8 followed by expression of BRG1, which together with Olig2 activates ZFP24 and CHD7 to regulate the onset of myelination and remyelination; (3) During onset of differentiation TCF7L2 interacts with ZBTB33 to repress β-catenin, which exerts a negative effect on myelination. (4) Recent work have also showed that dephosphorization of NFATC2, causes its activation and together with SOX10 prevents repression of Nkx2.2 and Olig2[133,134].

In parallel to differentiation, programmed cell death is extensive during development. Thus, in the developing rat cortex 20–30% of premyelinating oligodendrocytes (pre-OLs) undergo apoptosis prior to post-natal day 21 (P21)[128]. In the rat optic nerve as many as 50% of pre-OLs undergo apoptosis prior to myelination[135]. Two more recent and visually striking studies using *in vivo* imaging also show apoptosis to occur in adult rodents[136,137]. The reason for this temporal susceptibility to apoptosis is debated. The transcription factor TFEB-PUMA-axis, highly expressed in pre-OLs, has recently been indicated to be involved during development[138]. In addition, the same research group has suggested that loss of the platelet-derived growth factor (PDGF) receptor in pre-OLs, which is highly expressed in OPC, makes pre-OLs more susceptible to apoptosis[139]. Another plausible reason, that has recently been illustrated is that OLs receive trophic support from axons[249].

This is in line with studies showing that differentiating pre-OLs have a limited timeframe to make reciprocal contact with surrounding axons during development[140,141]. Upon proper trophic feedback in terms of basic fibroblast growth factor (bFGF), PDGF and/or insulin-like growth factor 1 (IGF-1) maturing OLs

will further differentiate and myelinate the naked axon[119,138,142–144]. During homeostatic conditions, OPCs and OLs will generate myelin throughout life, in mice up to two years of age[137,145]. However, in the absence of proper growth signals, programmed cell death programs will be activated[144]. One additional explanation for the temporal susceptibility of pre-OLs to apoptosis is suggested by the findings that OLs may display a maturation-dependent vulnerability to oxidative stress[146] (molecular mechanisms reviewed in section 1.21) and thus apoptosis[147]. Regardless of cause, the programmed cell death program in OLs involves both activation of caspases[148–151] and alternation of members of the B-cell lymphoma 2 (Bcl-2) family[146,152–154]. In summary, both OPCs and OLs seem to be dynamically regulated cell populations throughout the whole life.

1.41 Oligodendrocyte – form and function

OLs are essential since they provide insulation and trophic support to axons[155] and the axonal capacity to propagate signals is dependent on factors such as axonal diameter, myelin thickness, internode length and the distribution of nodes of Ranvier[156–158]. As mentioned above, the brain consumes a significant amount of oxygen to generate energy in order to maintain axonal resting potential, which is reflected by the fact that the brain stands for 20% of our total oxygen consumption. OLs are sensitive to ROS[159] especially during differentiation[146,148,160,161] and thus scavenging of these molecules is essential[146,162–166].

During limited periods of time, the brain can generate energy via anaerobic glycolysis producing pyruvate that enters the mitochondrial tricarboxylic acid cycle (TCA) in order to generate ATP. If not directly used by the mitochondria, lactate dehydrogenase (LDH) converts pyruvate to lactate for storage. Upon increased need of more ATP, LDH converts it back to pyruvate[167,168]. Lactate can also be directly imported to neurons via the monocarboxylate transporter 2 (MCT2)[167,168]. The glucose storage is restricted in neurons and thus neural glycolysis can only go on for shorter periods[169]. Instead the neurons have to rely on extracellular lactate supplied by adjacent and/or surrounding glia. In OLs, glucose is taken up by glucose transporter 1 (GLUT1) and converted to pyruvate and lactate. However, OLs can also export lactate for direct uptake by adjacent or surrounding neurons[170,171].

The importance of this extracellular lactate shuttling is elegantly shown by OL-specific monocarboxylate transporter 1 depletion causing severe axonal injury without damage to the OLs. Following administration of extracellular lactate the phenotype is reversed[171]. Interestingly, axonal swelling and degeneration appear to take place despite an intact myelin ultrastructure[170–172]. Instead, these studies suggest OL-specific loss of certain proteins involved in OL metabolic support to the axon as more crucial for axonal survival than the physical support from the myelin[170–172].

1.42 Remyelination

Regeneration of organs and/or tissues take place even in higher mammal species such as humans. In the skin, superficial lesions are healed and subsequently closed. The same concept applies for the brain where aOPCs traditionally are considered the principal source of myelinating OLs[145,173–175], repopulating demyelinated lesions[176–179]. This process, referred to as remyelination, is the fundament of proper regeneration of demyelinated lesions, e.g. in the context of neuro-inflammatory diseases such as MS.

In resemblance with skin-wounds leaving scars, remyelinated brain lesions can leave permanent[180] *shadow plaques*[181], first described by Lassmann *et al.* in 1983 (Fig. 4). Remyelinated axons will have longer internodes and thinner myelin sheaths, hence a reduced myelin staining and the appearance of a "shadow". Despite being thinner, the regenerated myelin protect the axon from degeneration[180,182–185]. Whether normal axonal function is preserved is more elusive.

Previous studies has shown preserved conduction and clinical functions despite thinner myelin[186,187]. However, there are also contradicting findings where computational approaches suggest that restoration of conduction will not be complete[188,189]. In addition, indirect evidence for reduced axonal velocity could be found in patients with hereditary motor and sensory neuropathies, suggestively due to the thinner myelin observed in these patients[190]. Likely restored velocity and normal axonal function following remyelination may depend on additional factors including axonal diameter and may also differ between tracts. With the assumption that demyelination as a primary event may cause secondary alterations in the axon, it has been shown that remyelinated OLs have a higher density of mitochondria compared to myelinated OLs[191]. This is supported by studies describing increased mitochondria activity in and transport along the axon in genetically or toxin-induced demyelination[27,28,192,193]. In addition, preliminary data of ours indicates a strong relationship between axonal mitochondria size and surrounding myeling sheath thickness[250]. However, what kind of molecular adaptions are needed in the axon and/or OLs to maintain sufficient axonal function following remyelination requires further exploration.

Based on studies conducted in rats, it is estimated that OPCs make up 3–8% of the total amount of CNS cells[194,195] and the OPCs are found throughout the brain, also in and around demyelinated lesions[196–198]. The involvement of OPCs during remyelination has been illustrated using Cre-based cell-fate mapping[199,200]. Upon need of regeneration, OPCs re-enter the cell-cycle[174,201–203]. Due to the presence of OPC, a block of differentiation has been suggested as the threshold preventing remyelination.

Interestingly, also other reasons explaining the relatively poor capacity to remyelinate in humans have been proposed[204]. A recent study conducted in cats and non-human primates showed that mature OLs can participate in remyelination[205] contradicting what has been shown in lower species, using Cre-recombinase under the Mbp-promoter[206]. Furthermore, two additional studies conducted with human cells, one of which combined single cell data from mice and MS patients, illustrate OLs to be more heterogeneous than previously expected[207]. The second study to some extent confirms that pre-existing mature OLs can contribute to remyelination due to a low degree of cell turn-over[208]. Together this underlines potential discrepancies in remyelination both between strains, species and experimental models.

Currently there is also a promising candidate for remyelination in clinical trials. Clemastine fumarate (CF) is an anti-histaminic drug identified to boost myelination in an *in vitro* screen[209–211].

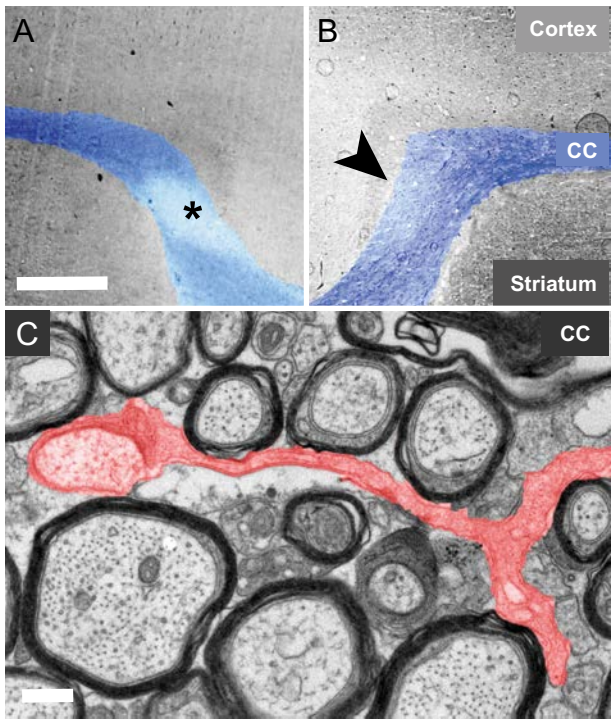


Figure 4. Myelin and oligodendrocytes in corpus callosum. *Representative images of Luxol fast blue stained and pseudo-colored corpus callosum.* ***(a)*** *Indicates a demyelinated area (asterisk),* ***(b)*** *indicates a shadow plaque (arrow).* ***(c)*** *Transmission electron microscopy image of transected axon with surrounding myelin and a pseudo-colored OL-branch myelinating an axon. Scale-bar a, b 500µm, c 1µm.*

2 METHODS

2.1 Flow cytometric measurement of ROS

Flow cytometric analyses are commonly used in the field of immunology to quantify chemical and physical characteristics of individual cells. This is made possible via fluorescent labeling of extra- and/or intracellular epitopes which upon excitation at the corresponding wavelength will emit fluorescent light that can be detected[212]. In Paper I flow cytometric analysis was used to quantify generation of intracellular ROS in $CD14^+$ monocytes using dihydrorhodamine-123 (DHR-123)[213,214]. In addition, proportions of monocyte subsets were also quantified[108,215] (Fig. 5).

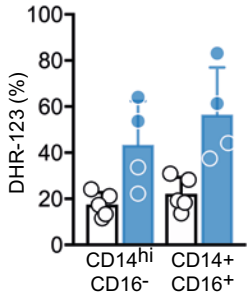


Figure 5. ROS generation in monocytes from MS-patients on DMF therapy. *ROS generation in CD14hiCD16- and CD14+CD16- monocytes before (white bars) and one month (blue bars) following DMF start.*

2.2 Animals

In Paper IV, based on previous findings by our group[216,217], a Dark agouti rat (DA^{Wt}) over-expressing Gsta4 under a CAG-promotor (DA^{Gsta4}) was purchased to study involvement of 4-HNE load in CNS cell survival and differentiation (Fig. 6). In addition, DA rats expressing green fluorescence protein (DA^{GFP}) and plain DA rats were used. Animals were bred in the animal facility at Karolinska University Hospital (Stockholm, Sweden) in a pathogen free and climate-controlled environment with regulated 12 h light/dark cycles. All experiments were approved and performed in accordance with Swedish National Board of Laboratory Animals and the European Community Council Directive. For all experiments animals at an age of 8–12 weeks were used. For all TBI (Section 2.3), LPC (Section 2.4) and bone marrow transfer in EAE experiments (Section 2.5) male animals were used. For regular EAE female animals were used.

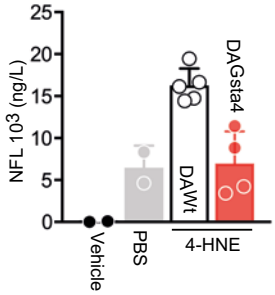


Figure 6. Resistance to intrathecally administered 4-HNE. *Neurofilament light (NFL) levels in rat CSF 24h following intrathecal administration of 4-HNE.*

2.3 Experimental traumatic brain injury model

The experimental traumatic brain injury (TBI) model used in Paper III is an open skull free falling weight drop model, representing a focal brain tissue injury[216–219]. There is a plethora of different experimental models for TBI generating different types of injuries. These can be separated based on the characteristics of the mechanical force and if generating focal versus diffuse injury following impact with subsequent effects on axonal degeneration which may be more diffusely distributed in the tissue versus affecting certain areas [220,221]. The model is characterized by an early infiltration of granulocytes and later activation of brain-resident microglia in combination with infiltrating monocytes[216–219] (Fig. 7). In humans, many victims of TBI display both focal and diffuse injuries. The model used here, represents mainly a focal injury model. The anesthetized rat is placed in a stereotactic frame followed by craniotomy (3 mm posterior and 2.3 mm lateral of Bregma). A piston is resting on the exposed dura and upon impact of the weight on the piston, the dura is compressed 3 mm on the longitudinal axis[44] (Fig. 7).

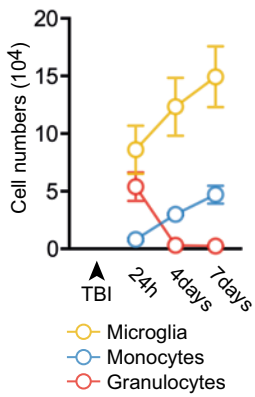


Figure 7. *Alternation in cell number following TBI in rats.*

2.4 Demyelinating lysolecithin model

Lysolecithin (lysophosphatidylcholine, LPC) is a chemical compound also present in low abundance in the cell membrane[222,223]. Upon apoptosis it partakes in the recruitment of phagocytes[224] and it is used to study remyelination, since it effectively removes a majority of mature OLs[225,226]. There are, however, other toxin induced models available to study remyelination[227]. Advantages of using LPC or ethidium bromide include the instant and focal demyelination of white matter around the site of injection, followed by consistent remyelination, a process that has been well documented. In Paper IV LPC was preferred due to its limited effect on astrocytes compared to ethidium bromide (Fig. 8). Administration of other commonly used toxins, such as cuprizone, also results in immediate demyelination, but requires a longer exposure time and thus demyelination and remyelination will occur simultaneously to a larger degree compared to LPC and ethidium bromide[179,227].

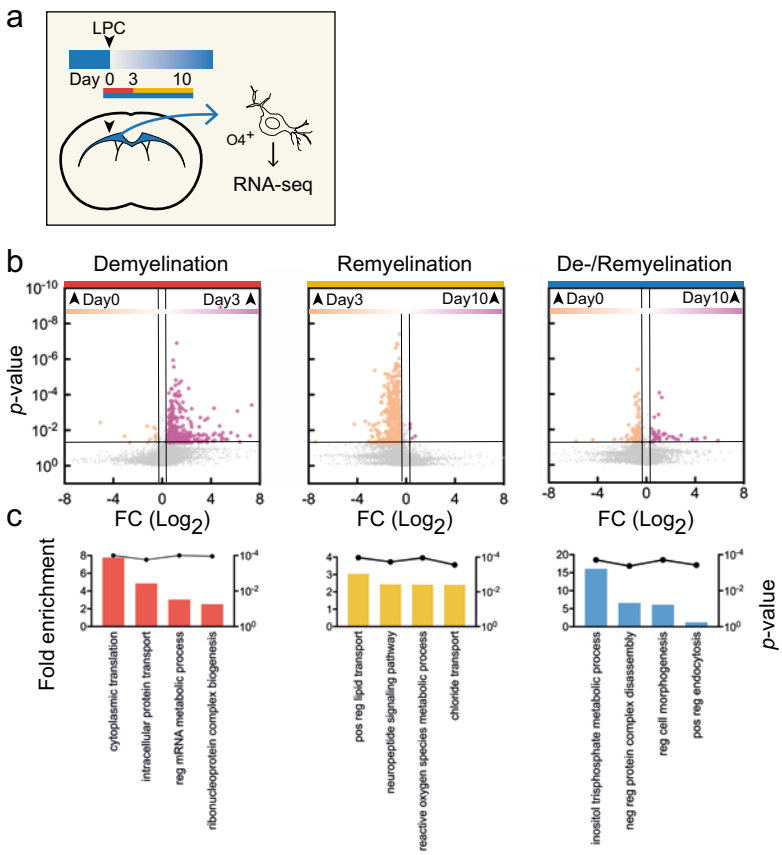


Figure 8. RNAseq of corpus callosum derived O4+ OLs during naïve state and three or ten days following LPC administration. ***(a)*** *Illustration of experimental set-up.* ***(b)*** *Significantly differentially regulated transcripts in DAWt during demyelination, remyelination and during both de- and remyelination.* ***(c)*** *Gene ontology based on significant transcripts in b.*

LPC can be administered to different white matter tracts[227] and herein we injected 25 µL 0.1% LPC to the CC, 1.0 mm posterior and 1.2 mm lateral of Bregma and 2.2 mm ventral of the skull bone.

2.5 Bone marrow transplantation and experimental autoimmune encephalomyelitis

In Paper IV we used a MS-like disease model, EAE. To a large extent our knowledge in MS, especially concerning cell trafficking and activation in the target tissue, is based on findings from EAE[94]. In rats, EAE is achieved by subcutaneous immunization with myelin oligodendrocyte glycoprotein (MOG) in a mixture with incomplete Freud's adjuvant (IFA). The adjuvant is necessary for the proper exposure of MOG to the local APC, which will migrate to local draining lymph nodes and present MOG-antigens to naïve $CD4^+$ T cells. These cells will then leave the lymph nodes and migrate to the CNS. The EAE model mimics several important features of the early relapsing-remitting disease phase of MS , however there are also limitations. This includes the need for active immunization, the lack of Fe^{2+} and oxidized phospholipids accumulation in CNS tissue seen in human, and absence of accumulation of oxidized phospholipids in active lesions. Also, active MS lesion pathology and EAE differ to a large extent in terms of gene expression of mitochondrial genes[228].

To study pathological processes in the spinal cord during EAE independently of genotype of the bone marrow derived immune cells we lethally irradiated animals and transplanted them with DA^{GFP} hematopoietic stem cells to repopulate the bone marrow.

3 AIMS

The overall aim of this thesis was to understand the various role of ROS and ROS-related biomolecules in different cells in demyelinating conditions.

Paper I

To characterize immune cell number, functionality, transcription and epigenetic changes following treatment with the DMF, targeting Nrf2 in RRMS patients.

Paper II

To develop a model to simultaneously measure responses of multiple intertwined transcription factors relevant for responses to ROS

Paper III

To identify novel Nrf2-specific drugs in comparison to DMF and evaluate those *in vitro* and in relevant experimental models.

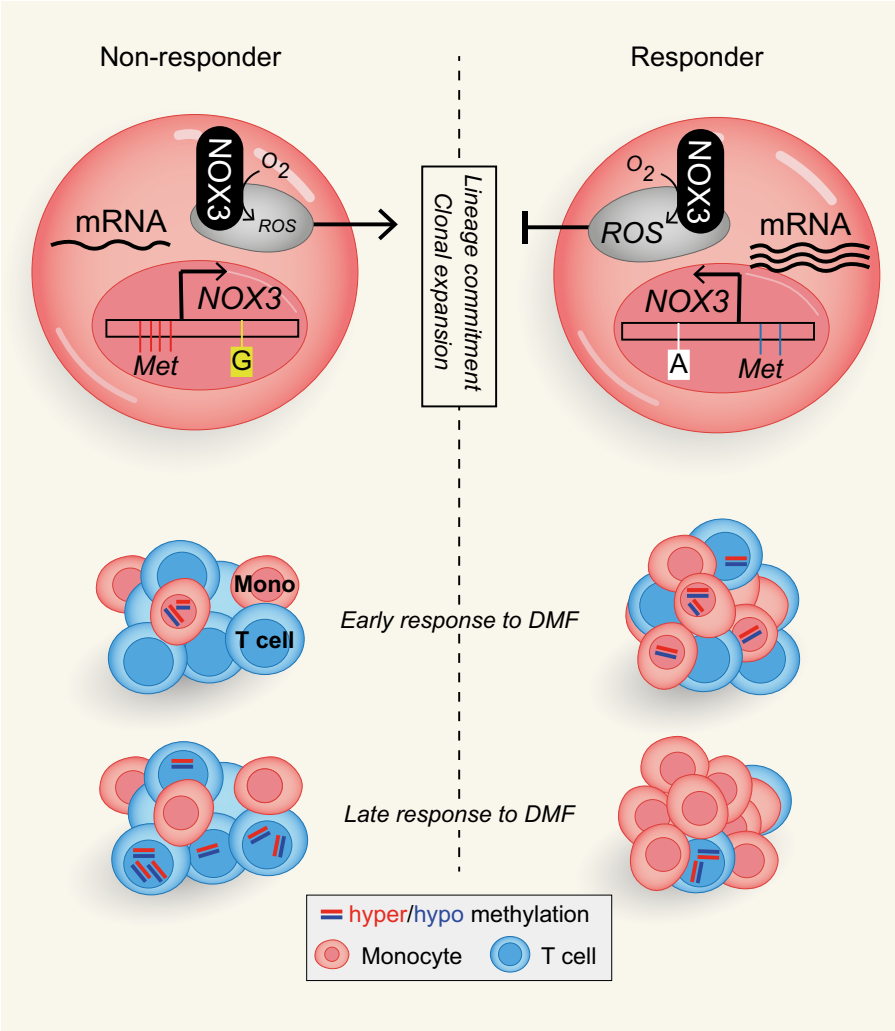
Paper IV

To assess the role of Nrf2-regulated Gsta4 during OL homeostasis and remyelination.

4 RESULT AND DISCUSSION

Paper I:

Therapeutic efficacy of dimethyl fumarate in relapsing-remitting multiple sclerosis is associated with ROS pathways in monocytes



In Paper I we addressed how DMF affects the oxidative environment in RRMS patients prescribed with DMF.

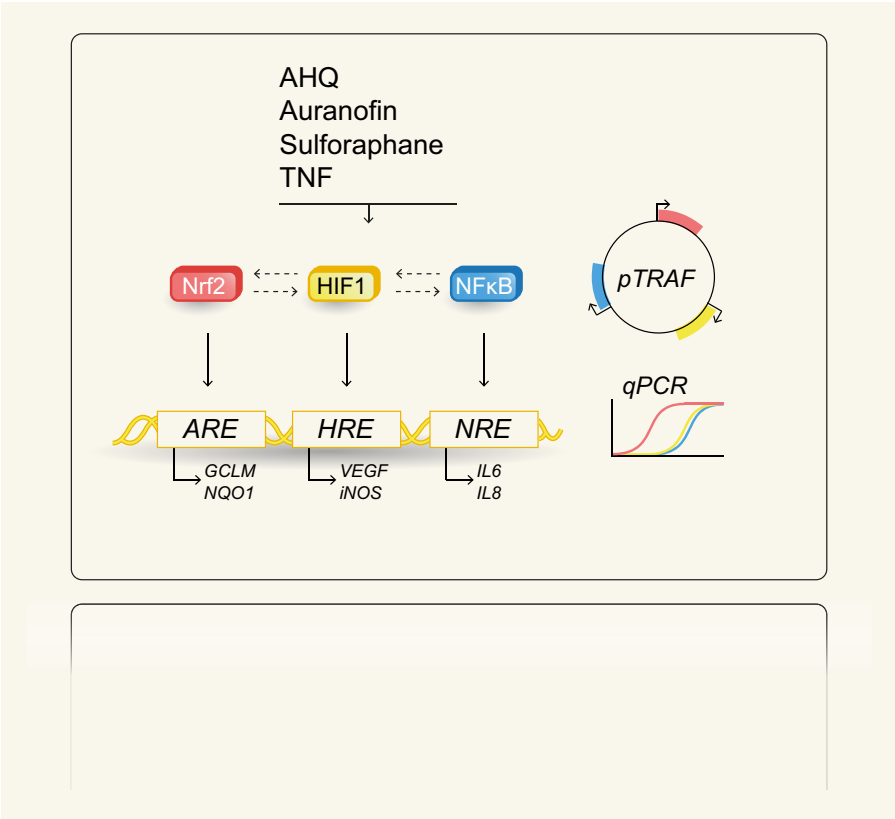
Based on existing knowledge of DMF we formed the hypothesis that DMF via Nrf2 were able to affect the oxidative environment in peripheral blood. We thus quantified ROS generation in peripheral monocytes and oxidation of free 8.12-iso-iPF2α-VI isoprostane in plasma from RRMS patients during their first 6 months on Tecfidera therapy. In addition, we sorted $CD4^+$ T cells and $CD14^+$ monocytes and assessed transcriptional and epigenetic changes over time. *DMF increased the oxidative environment in peripheral blood,* asses by free 8.12-iso-iPF2α-VI isoprostane. Patients showing no disease activity during the 24 months of observation were characterized as *responders*. Conversely, patients suffering relapses or inflammatory activity on MRI were characterized as *non-responders*. Interestingly, only *responders were able to increase their number of classical* monocytes and this difference was prominent already 3 months following therapy start. At this time point the monocytes from *responders were also capable of generating more ROS upon ex vivo stimulation* compared to DMF *non-responders*, this difference was also persisting after 6 months.

Characterization of DNA methylation changes over time further indicated that *epigenetic changes occurred earlier in monocytes compared to $CD4^+$ T cells.* Notably, in our cohort, monocyte numbers seemed to be an early marker segregating *responders* from *non-responders.* In contrast, lymphocyte numbers could not distinguish these two patients groups despite lowering of T cell number being a hallmark with DMF therapy[110,111]. The epigenetic data also indicated ROS to be involved in several pathways that were altered over time, which further supported our initial findings on changes in the oxidative environment. We further analyzed single nucleotide polymorphisms (SNPs) in a set of components to NADPH oxidases 1–4 expressed in at different levels in myeloid derived cells. One SNP in *NOX3* (rs6919626) was suggestively associated with reduced ROS generation after *ex vivo* stimulation, possibly due to lower transcription of *NOX3* in individuals carrying the G allele at rs6919626. Importantly, *SNP rs6919626 was also significantly associated with being a non-responder to DMF in terms of clinical effect.*

In summary, the generation of ROS in various immune cells is involved in regulation of autoimmune responses. This study builds on and extends a substantial body of pre-clinical evidence on how monocytes adapt to their endogenous ROS generation[229] and the central role of monocyte/macrophage-derived ROS on T cells[39,64,65,70,76]. Importantly, our findings in this study are the first to connect the capacity for oxidative responses to treatment responses in patients with RRMS. However, it should be noted that CNS-infiltrating monocytes are known to contribute to EAE progression[60], and the most abundant cell types in MS lesions are monocyte derived macrophages and microglia with damages due to ROS being evident in MS lesions[230]. Thus, the role of ROS for regulation of adaptive immunity and local target tissue responses may differ.

Paper II:

Cross-talk in HEK293 cells between Nrf2, HIF, and NF-κB activities upon challenges with redox therapeutics characterized with single-cell resolution



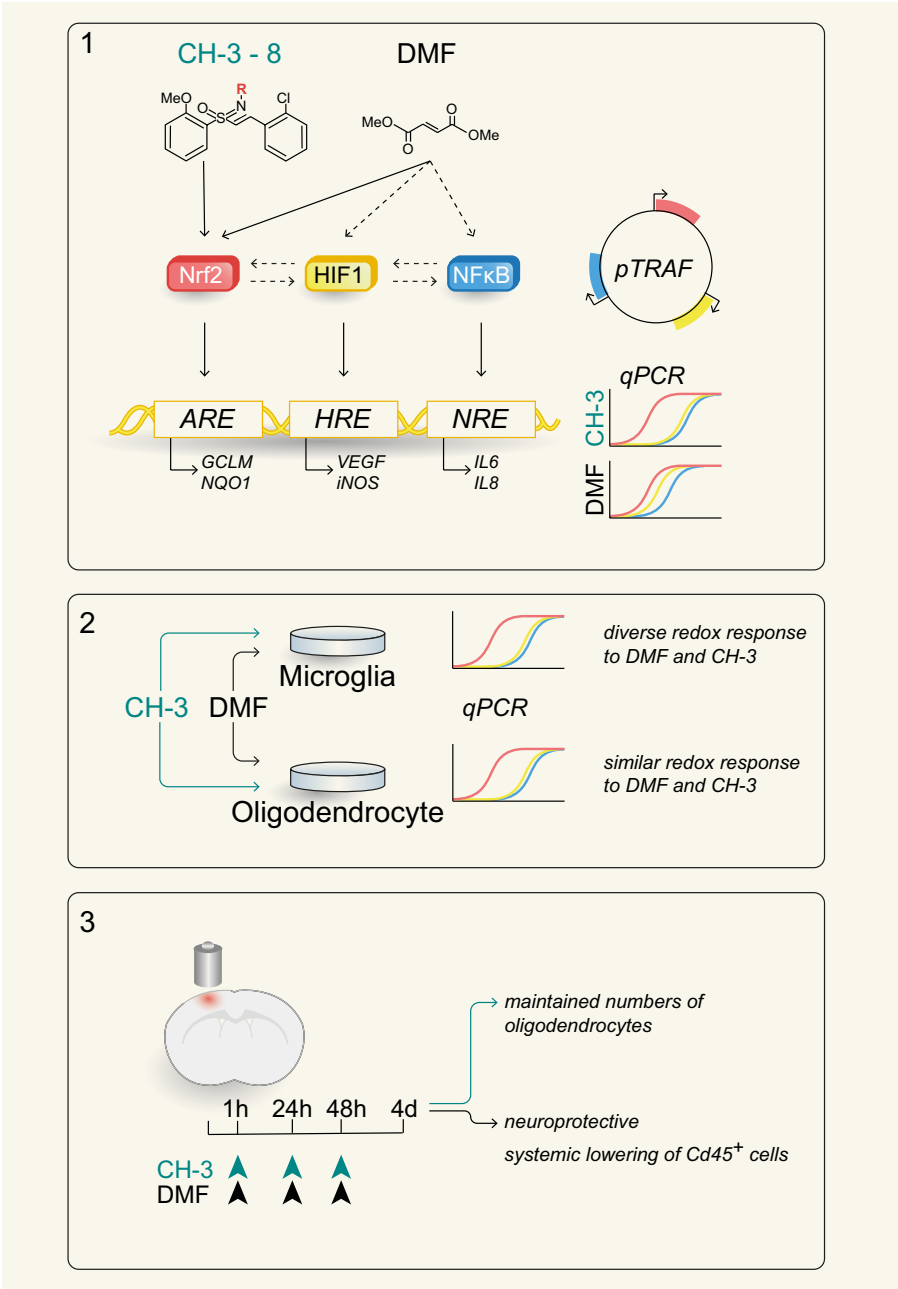
In Paper II our group assisted in the development of a novel method to detect transcription factor activity, namely *p*lasmids for *t*ranscription factor *r*eporter *a*ctivation based upon *f*luorescence (pTRAF) in human embryonic kidney cells (HEK293).

The activity of multiple transcription factors are intertwined during redox regulation. In this study, we developed and described a tool to study cross-talk between Nrf2, NFκB and hypoxia inducible factor (HIF) in real-time at the single-cell level and in a high throughput manner. As discussed in section 1.2, Nrf2 regulates a plethora of transcripts involved in responses to oxidative stress. Upon activation, Nrf2 is relocated to the nucleus, promoting transcription via binding to ARE in target gene promoter regions (Fig.1). HIF is made up by heterodimers of HIFα and HIFβ subunits and promotes transcription in response to hypoxia. NFκB is a complex of subunits that initiates inflammatory and cellular responses to various cytokines and stressors. IκB keeps NF-κB in the cytosol, but if phosphorylated, NF-κB is released and able to relocate to the nucleus. Based on previously established gene binding regions of the three transcription factors[231,232], vectors expressing *lucP2* upon binding of Nrf2, HIF or NF-κB in a Luciferase assay were generated and verified to bind the respective transcription factor. Following this, a single vector containing all binding regions followed by sequences for *mCherry* (Nrf2), *YPet* (HIF) or *CFP* (NF-κB), respectively, were generated.

Upon transfection of HEK293 cells with pTRAF, transcription factor binding generated a fluorescent signal, and the factual generation of transcripts corresponding to Nrf2/HIF/NF-κB activity was confirmed with quantitative polymerase chain reaction (qPCR). This included *GCLM* and *HMOX1* (Nrf2), *HIF1A* and *VEGF* (HIF), and *IL8* (NF-κB). Based on the feature of simultaneous Nrf2/HIF/NF-κB-response with single-cell resolution, cells following transfection were quantified and sorted using flow cytometry. In addition, analyses in Paper II were done using Operetta high-content imaging allowing for *high-throughput analysis of Nrf2/HIF/NF-κB-responses to various stimulations*.

Paper III:

Novel vinyl sulfone compounds are more specific Nrf2 activators in vitro and in the central nervous system than dimethyl fumarate



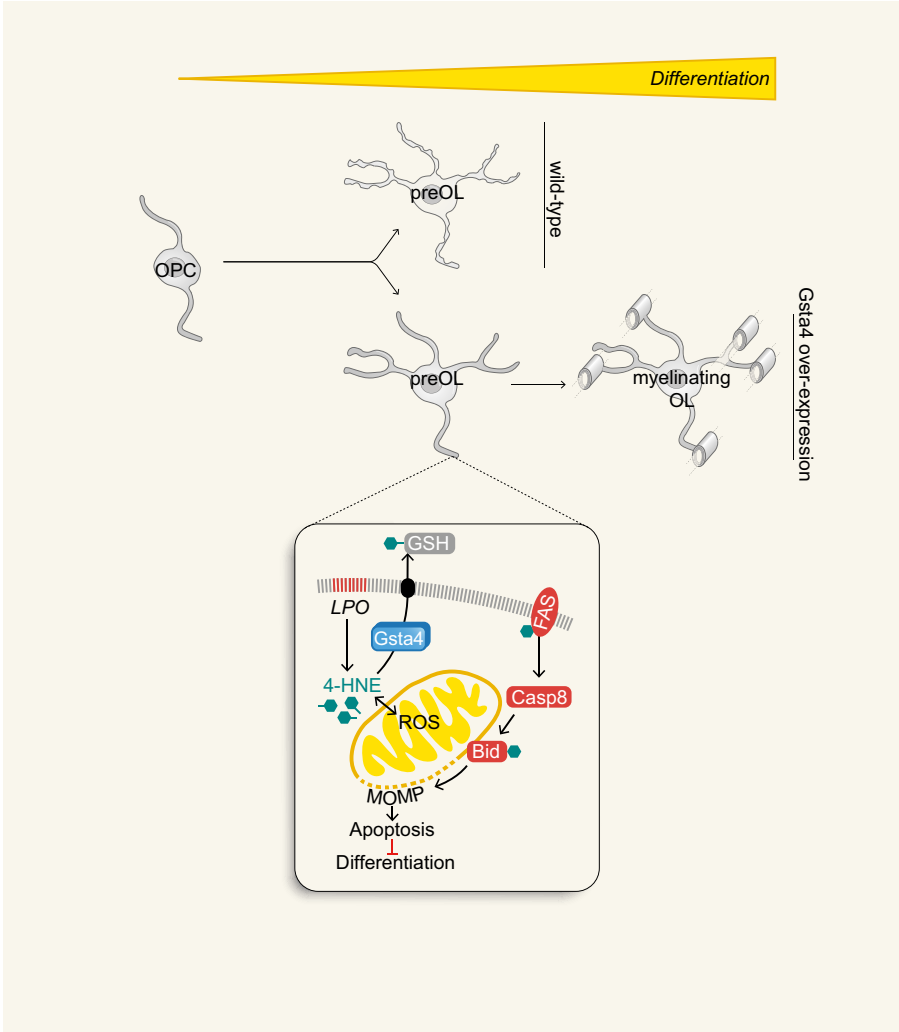
In Paper III we evaluated DMF together with additional newly synthetized Nrf2 activating compounds based on their activity towards Nrf2 and other transcription factors described to be affected by DMF.

Based on previous publications, we formed the hypothesis that addition of certain chemical moieties would improve Nrf2 specificity and improve stability and passage over the BBB[233–238]. We thus evaluated DMF together with eight *de novo* synthetized vinyl sulfone compounds CH-1 – CH-8 based on cell viability and ability to activate Nrf2. CH-1 – CH-3 were the most promising candidates and were further evaluated based on their activity towards NFκB and HIF, which has been described to be affected by DMF[114]. *Both DMF and CH-3 activated Nrf2 but CH-3 showed less off-target on NFκB or HIF.* This was also verified by quantification of transcripts downstream of the three transcription factors. Nrf2 is activated in both microglia and OLs upon stimulation with either vinyl sulfone[239,240] or DMF[104]. Thus, the transcriptional profile was evaluated in these cell-types following CH-3 or DMF.

Nrf2, HIF and NFκB as assessed by pTRAF in HEK293 cells, are also key transcription factors in conditions of acute trauma and thus CH-3 and DMF were finally evaluated in an experimental TBI model[43]. *CH-3 facilitated more extensive transcription of Nrf2-regulated genes in the brain compared to DMF upon oral gavage, while* DMF induced an immediate drop in GSH levels as reflected by *Gclm* and *Gsta4*[241]. Following TBI in combination with oral gavage with vehicle, DMF or CH-3, DMF (but not CH-3) lowered systemic $CD45^+$ cells as previously reported[110,111]. In addition, *DMF also limited axonal degeneration following TBI compared to vehicle.* In turn, CH-3 preserved or facilitated proliferation and differentiation of OLs following TBI compared to DMF and vehicle. In summary, our findings indicate that CH-3 differ from DMF in its effect towards Nrf2, HIF and NFκB and their downstream transcripts and that Nrf2-mediated effects are largely responsible for transcriptional changes occurring in OLs.

Paper IV:

Gsta4 restricts apoptosis to promote differentiation of adult oligodendrocytes via Fas/Casp8/Bid-axis during homeostasis and remyelination



In Paper III we evaluated Nrf2 activating compounds including DMF and CH-3. DMF (Tecfidera) is a known inducer of gene transcription downstream of Nrf2[103,104].

Previous work in our research group explored the role of Glutathione S-transferase 4α (Gsta4) in neuroprotection during acute inflammation in the brain[216,217]. In addition, Gsta4 has been a predicted transcript downstream of the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)[6,242], which is the suggested main target of DMF used to treat RRMS[100,103,104] (Fig. 1). In Paper IV we investigated the impact of high transitional levels of Gsta4 in rat OLs during differentiation and remyelination.

The underlying cause for poor remyelinating capacity of OL in MS is still largely unknown. Lack of knowledge in this area severely hampers the development of treatments that enhance remyelination. Due to the relatively high susceptibility of OLs to ROS and lipid peroxidation[146,160,162,166], which generates 4-HNE, we formed the hypothesis that over-expression of the 4-HNE scavenging enzyme Gsta4 could provide a beneficial intracellular environment in OLs during differentiation and remyelination. We found that both remyelinating drug, *clemastine fumarate (CF)*[210,243,244] *and DMF increased Plp and Gsta4 transcription,* the former in a Gsta4-dependent manner and the latter due to nuclear activation of Nrf2. Creation of a transgenic rat with constitutive over-expression of Gsta4 (DA^{Gsta4}) facilitated faster OL differentiation *in vitro* compared to wild-type (DA^{Wt}), a phenotype that was reveres upon Gsta4 knock-down. The faster differentiation was verified *in vivo* as *DA^{Gsta4} displayed a drastic reduction in OPC number and early OLs,* while DA^{Wt} and DA^{Gsta4} did not differ in number of mature OLs.

RNA-seq on mRNA from $O4^+$ OLs from the CC indicated the Fas/Caspase-8 pathway and bFGF signaling to be reduced in DA^{Gsta4} OLs and likely responsible for the faster differentiation. This was confirmed as stimulation of DA^{Wt} OLs with a Fas-antagonist promoted differentiation to a similar extent as in DA^{Gsta4}. In addition, both *Caspase-8 and the downstream target and inducer of apoptosis Bid were decreased upon Gsta4 over-expression* and increased upon knock-down. Furthermore, in a toxin-based demyelination model of the CC higher levels of *Gsta4 led to faster remyelination.* Finally, in the MS-like disease model EAE, *DA^{Gsta4} animals showed improved clinical severity score and disease duration compared to DA^{Wt}.* When lethally irradiating and transplanting DA^{Wt} and DA^{Gsta4} rats with identical DA^{GFP} bone marrow, $DA^{GFP}\rightarrow DA^{Gsta4}$ again showed improved clinical severity score and disease duration compared to $DA^{GFP}\rightarrow DA^{Wt}$. In line with previous experiments, $DA^{GFP}\rightarrow DA^{Gsta4}$ showed *lower levels of Caspase-8 in spinal cord but also smaller and fewer demyelinated lesions, likely due to improved remyelination seen in DA^{Gsta4}.*

Pioneering studies identified bFGF to be essential for OL development[140,143] and described cell-cell interaction as crucial for OL survival[135,144]. A more recent study underlines the vulnerability of OLs during differentiation as they only have a few hours to generate myelin[141]. In summary, our study indicates that newly formed/myelin forming OLs to represent a particularly sensitive state during OL maturation and that the OLs resistance to apoptosis and/or facilitation of more efficient differentiation can be enhanced via over expression of Gsta4. This study also indicates Gsta4 and associated mechanism, including the Fas/Caspase8/Bid-axis, to be highly interesting in context of the mode of action of certain remyelination-promoting agents.

5 FURTHER PERSPECTIVES

Today we have access to a number of highly effective immunomodulating treatments in MS that almost entirely can prevent new bouts of disease and drastically improve the long-term prognosis. However, these treatments are most effective in the initial phases of MS and many patients in later disease phases suffer continued disease worsening. The striking advances in treatment of early RRMS in part depends on improved knowledge of underlying disease processes, while the understanding of disease mechanisms operating in progressive MS remains poorly defined. MS involves the loss of neurons, the replacement of which represents a monumental challenge. In contrast, enhancing remyelination by modulating existing OPCs or perhaps even OLs could be a more tempting target.

This picture is supported by the observations we made in the included studies. We herein show that support to differentiating cells to overcome certain thresholds or crossroads during differentiation in rodents is beneficial in MS-like disease models. Our findings indicate the importance of redox processes in overcoming these thresholds and this could also be of importance in humans. We further show that redox balance is a key-element in additional aspects of MS disease where location and cell type is decisive of the outcome. In addition we show that its feasible to identify more specific Nrf2 activating drug compared to the currently used drug.

On a personal note, it is a captivating time to study oligodendrocytes. A string of recent discoveries have revealed the heterogeneity and plasticity of OL cell lineages during maturation[137,245]. In addition, participation by non-dividing OLs in remyelination in higher species brings a new perspective to the field[205,208]. In particular I have identified two fields of high interest that deserve further exploration.

Characterization of temporal metabolic changes during OL maturation. By tradition, metabolism in OLs has been addressed from a nerve cell-perspective, e.g. how OLs are able to maintain axonal function and integrity via shuttling of lactate and glucose between the cells. However, metabolic fluctuation during different OL maturation states is not described. Especially halted maturation in context of demyelinating disorders is an intriguing phenomenon. Metabolites, such as lactate, have recently been described to also possess epigenetic modulatory features by inducing histone modifications; a process termed lactylation[246]. Since OLs are crucial for axonal lactate maintenance the role of OLs also in neural lactylation is an interesting research field.

Additional in vivo models of remyelination. Lately and partly due to technical advances it has become clear that there are crucial discrepancies in remyelination between human and lower species[247], with consequences for our interpretation of earlier experimental data and to what degree this can be translated to humans. Neumann *et al.* have addressed the interesting question how ageing of adult OLs may affect their phenotype[132]. Nobuta *et al.* likewise have studied OL death in PMD[248]. Both these studies benefit of their (in comparison) simple set-up to study failure of OL maturation. To study OL maturation arrest in alternative models could be very useful and could later lay the foundation for applications in other diseases, such as MS.

6 ACKNOWLEDGEMENT

I want to thank my good friend and supervisor (and physician) *Fredrik*. I have learned a lot from you over the years. You have an inspirational scientific eye, a critical mind and honest willing to support your students. On a personal level you are caring and you have my deepest admirations for operating a fruitful research-carrier and simultaneously be with the family=). I want to thank my co-supervisors *Faiez* for introducing me to the animal-models and giving me a solid professional foundation. *Xingmei*, for your care and support, you always brought joy to the lab and miss chatting with you. *Staffan*, thank you for your support in our initial pilot experiment.

I'm grateful to *Ana* for being the crucial factor when we were setting up OL experiments. Also *Gonçalo* for valuable experiment guidance and assistance. Thank you *Katarina* for always having time for questions and time to discuss and explain redox biology. I was never able to pass by your office just for a brief stop, and I appreciate that a lot. Also, thanks to *Elias*, *Per, Craig* and *Toni* for a good collaboration in various projects. Thank you everyone at the Birsoy Lab especially *Rebecca*, *Javi* and *Kivanç.*

Thank you *Tomas, Ingrid, Maja* and *Bob* for the creation of a nice and stimulating environment over the years. You have all been inspirational and good collaborators over the years.

I also want to thank certain teaches that I luckily have met; *AnnMarie* and *Henrik* for getting me interested in biology and chemistry at senior high. Thank you *Christine* in Lund for a good introduction to neuroscience. I'm also grateful to *Mattias* in Lund being a mentor and letting me in your lab during the summers.

A period during my PhD was the worst time of my life, but foremost, I have also experienced the best moments of my life during this period. I'm forever grateful for all the people I've met and a will always remember my time in this awesome environment. To the original members of the man-cave: *Mohsen*, this place wouldn't work without you. *André* our MVP, you are brilliant and I'm so glad that I have got to know you. I'm happy for may close friendship with *Petra, Harald* and *Rasmus*. We have enjoyed a lot of things together and I will always remember our travels, dinners parties…

A also want to thank all present members at the lab. *Lara* and *Eliane* you are both truly kind and caring souls and I will truly miss not having you around on an everyday basis. Thank you *Rux* and *Nånnis* for being such bright scientists and with the best sense of humor. My greatest gratitude to *Keying,* helping out with OLs and being a bright young man. Thank you *Sandra* and *Ewoud* for being patient with

(with me) and our patient data. I'm very grateful for all project input, all scientific and general life discussions with *Benjamin, Sreeni, Susi, Rita, Jinming, Majid, Gunn, Pernilla, Maria, Mathias, Galina, Sebastian.* Thank you to former master students *Emina* and *Inge* and good luck with your defence.

A big thank you to past members, *Cecilia*, for being a good first supervisor. Also, *Mikael, Rickard, Shahin, Nada, Tojo, Sabrina, Melanie, Roham, Marie*.

A big thank you to all staff in the animal facility helping me out throughout the years. Especially *André, Annica, Sandra, Anna-Lena* and *Velina* for taking excellent care of breeding and your ability to always allocate space for some additional cages=) Also a big thank you to *Anki* assisting with ethical permits.

Thank you *Mom* and *Dad* for always supporting me, my sister and my family in whatever we do and for being interested in my work. Thank you *Klara* for being the best sister.

Last but not least I would like to express my greatest gratitude to *Therese, Assar* and *Iris*. For bringing me joy every day (currently not during the nights). For putting up with this job/hobby of mine. I'm dearly grateful and you have been nothing but supportive and willing to share all my professional ups and downs with me. Tack!

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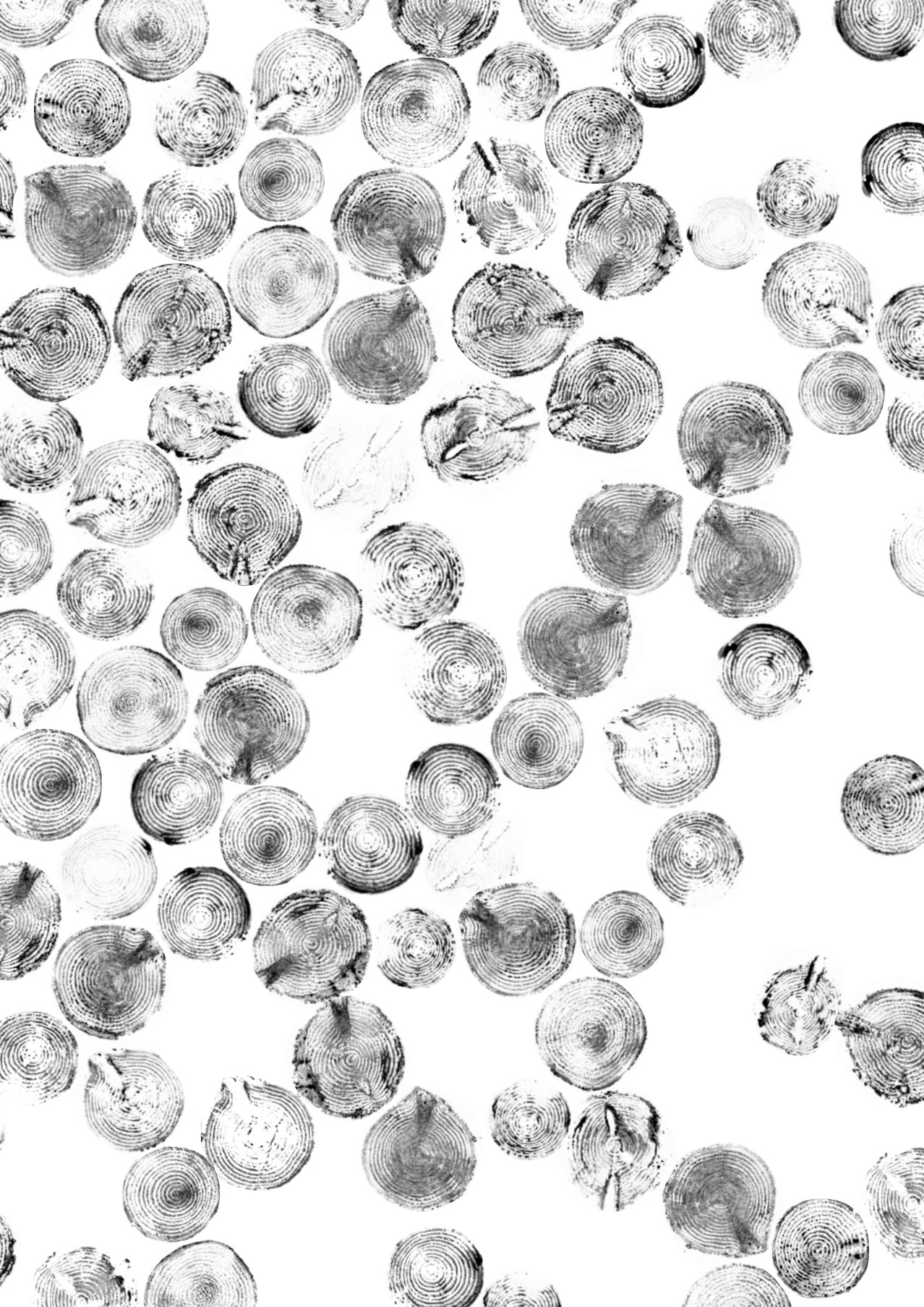

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Arkitektkopia AB, Bromma, 2020