Investigation into the epigenetic mechanisms involved in microglial activation in the animal model of multiple sclerosis

Karla G. Lam Haces Gil
Green-Templeton College, University of Oxford
A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Hilary term, 2013

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Abstract

In patients with multiple sclerosis (MS), microglia become activated due to the autoimmune inflammatory response which is directed against the central nervous system (CNS). Following the first disease relapse, microglia remain activated and do not return to a resting state during remissions. Chronically-activated microglia release inflammatory mediators that cause CNS tissue damage, and as such, MS progression has been associated with widespread, chronic microglial activation that correlates with neurodegeneration. To date, only one histone demethylase, Jmjd3, has been described to have a role in inflammation. In agreement with this, up-regulation of Jmjd3 expression was observed following microglial treatment with several pro-inflammatory stimuli, including a range of toll-like receptors ligands and cytokines, suggesting a universal role of Jmjd3 during microglial activation. Subsequent ChIP-qPCR assays revealed that Jmjd3 was recruited to the promoters of *Il6*, *Ccl3*, *Ccl5* and *Nos2* following activation, which, in turn, presented a decrease in their H3K27me3 levels. Using an experimental autoimmune encephalomyelitis (EAE) mouse model of MS, Jmjd3 expression was shown to be increased in activated microglia from mice in the acute and late phases of disease. Immunization with complete Freud's adjuvant (CFA) alone, also caused microglial activation with Jmjd3 induction, indicating a CFA-mediated TLR2 and TLR4 stimulation of microglia. Further investigation, in which primary microglia were isolated from mice deficient in Jmjd3 (*Jmjd3*$^{-/-}$), however demonstrated that the absence of Jmjd3 alone had no resultant effect on the expression of a subset of immune response and inflammation related genes, including the Jmjd3 target genes *Il6*, *Ccl3*, *Ccl5* and *Nos2,* before or after activation. This suggested that Jmjd3 acts in concert with a repertoire of other demethylases to facilitate microglia activation, and as such was rendered redundant in this setting. Deciphering the epigenetic profile of microglia in MS and determining whether it is involved in the maintenance of chronic microglial activation in the progressive phase of the disease remains an important line of investigation, and through a clearer understanding of its role in MS pathophysiology, could lead to the development of novel therapeutic interventions in the future.

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List of abbreviations

15-HC	15α-hydroxicholestene
ACM	Astrocyte-conditioned media
APC	Antigen presenting cell
ANCA	Anti-neutrophil cytoplasmic autoantibody
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
Bmp-2	Bone morphogenetic protein 2
bp	Base pair
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
ChIP	Chromatin immunoprecipitation
ChIP-qPCR	Chromatin immunoprecipitation-quantitative real-time polymerase chain reaction
CNS	Central nervous system
CpG	Cytosine-guanosine
CSF	Cerebrospinal fluid
CR3	Complement receptor 3
CR	Chronic-relapsing
CR-EAE	Chronic relapsing-experimental autoimmune encephalomyelitis
CSF	Cerebrospinal spinal fluid
CTD	Carboxy-terminal domain
DAMP	Damage associated molecular pattern
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl sulphoxide
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ES	Embryonic stem
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GBP6	Guanylate-binding protein 6
GFP	Green florescence protein

H3	Histone 3
H3K4	Histone 3 lysine 4
H3K9me3	Histone 3 lysine 9 trimethylation
H3K27me3	Histone 3 lysine 27 trimethylation
H3K36	Histone 3 lysine 36
H3K79	Histone 3 lysine 79
HBSS	Hanks' balanced salt solution
HDAC	histone deacetylase
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HMT	Histone methyltransferase
HPK1	Hematopoietic progenitor kinase 1
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
HSP60	Heat shock proteins 60
IFN	Interferon
IL	Interleukin
IL2RA	Interleukin 2 receptor alpha
IL7R	Interleukin 7 receptor
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
JmjC	Jumonji C
αKG	α-ketoglutarate
KIF1B	Kinesin family member 1B
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
Mac-1	Macrophage-1 antigen
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatability complex
MIP-1α	Macrophage inflammatory protein 1 alpha
MOG	Myelin oligodendrocytes glycoprotein
MPO	Myeloperoxidase

MS	Multiple sclerosis
MRI	Magnetic resonance imaging
MyD88	Myeloid differentiation factor 88
NAWM	Normal-appearing white matter
NGS	Normal goat serum
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
PAD2	Peptidyl arginine deiminase type-2
PAMP	Pathogen-associated molecular pattern
PARP-1	Poly (ADP-ribose) polymerase I
PBC	Pre-Bötzinger complex
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
PKR	Protein kinase dsRNA-regulated
PLP	Proteolipid protein
PMφ	Peritoneal macrophage
Pol II	RNA polymerase II
PT	Pertussis toxin
PR3	Proteinase 3
PRC2	Polycomb-repressive complex 2
PVM	Perivascular macrophage
qRT-PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptors for advance glycation end product
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SAS	Sub-arachnoid space
SIP	Stock isotonic percoll
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TGF-β	Transforming growth factor-β
T_H	T helper
TIR	Toll–interleukin 1 receptor
TLCK	Na-Tosyl-L-lysine chloromethyl ketone hydrochloride

TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR1A	Tumour necrosis factor receptor 1 alpha
TRIF	TIR-domain-containing adaptor-inducing interferon-β
TSS	Transcription start site
TU	Transduction units
TYK2	Tyrosine kinase 2
WT	Wild type

1. Introduction

1.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune, chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) that targets the myelin sheath and oligodendrocytes. It constitutes the leading cause of progressive neurological disability in young adults in the Western world (Compston and Coles, 2008).

1.1.1. MS etiology

MS is a multifactorial disease with both environmental factors and genetic susceptibility implicated in its etiology (Compston and Coles, 2008; Noseworthy et al., 2000).

1.1.1.1. Genetic factors

Genetic susceptibility has an essential role in the development of MS. The importance of genetic factors is demonstrated by an approximate 30% concordance rate in monozygotic twins and by a 10 to 25 times higher risk of developing MS in first-degree relatives of MS patients when compared to the general population (Robertson et al., 1996; Sadovnick et al., 1993; Sadovnick et al., 1988).

Human leukocyte antigen (HLA) gene variants (alleles) have the strongest genetic effect in MS (Oksenberg et al., 2008). The major contribution to disease risk is conferred by the HLA-DR2 haplotype, a major histocompatability complex (MHC) class II region that contains the alleles HLA-DRB1*1501, HLA-DRB5*0101 and HLA-DQB1*0602, which are most commonly inherited together in a block (linkage disequilibrium) (Olerup and Hillert, 1991). Of these alleles, HLA-DRB1*1501 (encoding HLA-DR2b) is the main susceptibility allele in MS (Oksenberg et al., 2004). The HLA-DRB1* 0801 allele and the HLA-DRB1*1401 allele confer

higher and lower MS risk respectively, if carried by HLA-DRB1*1501 positive subjects (Barcellos et al., 2006). The MHC class I region also contains susceptibility loci. The HLA-A*0301 allele predisposes individuals to disease while HLA-A*0201 and HLA-C*05 show a protective effect (Brynedal et al., 2007; Fogdell-Hahn et al., 2000; Yeo et al., 2007).

More recently, genome wide association studies have identified added gene variants that confer minor risk of developing MS. These include single nucleotide polymorphisms at immunological and neurological genes such as interleukin receptor 2 alpha (IL2RA), interleukin receptor 7 (IL7R), tumour necrosis factor receptor 1 alpha (TNFR1A), tyrosine kinase 2 (TYK2; a signal transduction molecule for various cytokines), and kinesin family member 1B (KIF1B; an axonal transport protein) among others (T.I.M.S.G.C.T.W.T.C.C.C, 2011).

1.1.1.2. Environmental factors

Environmental factors substantially influence the development of MS in genetically susceptible individuals.

The incidence and prevalence of MS increase with distance from the earth's equator on both sides (Kurtzke, 1993). Genetic and ethnic factors partially account for this distribution, since MS is common within northern Europeans, however latitude is the strongest factor for risk after controlling for ethnicity (Wallin et al., 2004). Sunlight exposure and vitamin D levels have been suggested as a possible explanation for the influence of latitude in MS incidence, as past sunlight exposure and serum levels of the active form of vitamin D are inversely related to MS susceptibility (Mei et al., 2003; Munger Kl, 2006). Moreover, there is a correlation between the risk of MS and the place of residence before adolescence. Immigrants that move residency during childhood acquire the risk of the new region while the ones that migrate later in life retain the risk of their country of origin (Dean, 1967; Dean and Elian, 1997).

Therefore, it seems like environmental exposure early in life can increase susceptibility to MS (Ramagopalan et al., 2010).

Viral infections have been linked with an increased risk of developing MS, including Epstein-Barr virus, rubella, and human herpesvirus 6 among others (Andersson et al., 1994; Kurtzke, 1993; Levin Li and et al., 2003; Soldan et al., 1997; Thacker et al., 2006). However, it seems that delayed infection in life rather than a unique infection contributes to disease development by causing abnormal immune responses (Ascherio and Munger, 2007; Ramagopalan et al., 2010). Infectious agents might also provide the appropriate conditions for the start of an autoimmune response against the CNS (Frohman et al., 2006). Pathogens stimulate toll-like receptors (receptors that recognise pathogen-associated molecular patterns) in the surface of innate immune system cells and promote the secretion of pro-inflammatory cytokines, including IL-12 and IL-23, that might contribute to the differentiation of naïve autoreactive $CD4^+$ T cells into effector cells (Waldner et al., 2004). Systemic infections could also activate the cerebrovascular endothelium and up-regulate the expression of adhesion molecules necessary for the migration of autoreactive T cells into the CNS (Kerfoot et al., 2004). However, Epstein-Barr virus and enterobacteria might actually initiate MS in susceptible individuals through molecular mimicry with myelin antigens. These pathogens possess peptides that have a similar amino acid sequence or molecular structure with myelin self antigens. This causes T cell responses to the infection that cross-react with myelin, producing CNS inflammation and demyelination (Harkiolaki et al., 2009; Lang et al., 2002).

Other environmental factors that have been involved in MS are toxins found in cigarettes and pollution (Hawkes, 2007; Marrie, 2004).

1.1.2. Clinical course

MS presents three main clinical courses: relapsing-remitting (affecting 80% of patients), secondary progressive and primary progressive (Lublin et al., 1996). Relapsing-remitting MS (RRMS) has an average age of onset of approximately 30 years and is around two times more common in women than in men (Orton et al., 2006; Weinshenker et al., 1989). Around 80% of patients debut as a clinically isolated syndrome, which is the first neurologic episode with clinical manifestations suggestive of MS. The chance of developing RRMS is 50% at 2 years and 82% at 20 years if the clinical episode is accompanied by white matter abnormalities in the magnetic resonance imaging (MRI) (Fisniku et al., 2008). In the early phase, relapsing-remitting patients have further acute episodes (relapses) in which symptoms and signs develop over the course of several days, become stable and then improve within weeks (remission). These symptoms and signs include limb weakness, sensory disturbances, fatigue, optic neuritis, diplopia, gait ataxia and bladder and bowel dysfunction, among others. Later in the disease, cognitive impairment, spasticity, depression, dysarthria (speech difficulty), dysphagia (impaired swallowing), sensory loss, progressive quadriparesis and tremors are also added to the clinical presentation. Over time, the recovery after episodes becomes incomplete and the patients present persistent and progressive signs of CNS dysfunction with increasing motor, sensory and autonomic disability (secondary progressive MS). Primary progressive patients show a progressive disease course from the onset (Compston and Coles, 2008; Noseworthy et al., 2000).

1.1.3. Mouse models of MS

Given the limited availability of CNS tissue from MS patients, especially from those in the active stage of disease, MS research has largely relied on an animal model of experimental

autoimmune encephalomyelitis (EAE) to elucidate the molecular and inflammatory mechanisms of the disease (Gold et al., 2006).

EAE models were originally developed after the observation that multiple injections with emulsified brain and spinal cord caused signs and symptoms similar to MS in rabbits and monkeys (Rivers et al., 1933). It was later seen in rabbits that the more antigenic the emulsion administered, the higher the antibody titer and the number of affected rabbits. This allowed researches to establish an association between brain-specific antibodies and demyelination, and also to hypothesise that the disease was caused by a targeted immune response (Schwentker and Rivers, 1934). To try to enhance the response to sensitisation with CNS homogenates, different research groups started using a mineral oil-based adjuvant developed by Jules Freund to which heat-inactivated mycobacterium tuberculosis was added (complete Freund's adjuvant, CFA). The use of adjuvant allowed the induction of autoimmune encephalomyelitis in a wider range of species with only a few injections of CNS emulsion (Freund et al., 1947; Kabat et al., 1947).

Since then, EAE has been induced in guinea pigs, goats, mice, rats, hamsters, dogs, sheep, marmosets and chickens. The clinical features and the histopathology of the disease vary significantly between experimental protocols, depending on the mode of induction, the immunizing antigen and the animal model used. Diverse, well characterised rodent and primate models that imitate different immunopathological aspects of MS have been developed (Baxter, 2007; Gold et al., 2006). However, in general EAE displays histopathological characteristics that resemble those of MS including: different degrees of myelin destruction and widespread axonal damage, the presence of multiple CNS lesions distributed in time and position with a predominant perivascular location, the transition from inflammation and demyelination to gliosis and partial remyelination observed in the lesions, and the presence of

immunoglobulin in the CNS and cerebrospinal fluid (only in some models) (Adams and Kubik, 1952; Brown et al., 1982; Wolf et al., 1947).

Nowadays, mice and rats are the most commonly used species for the induction of EAE because of their easier care and husbandry and because of the availability of genetically modified specimens in the case of mice (Gold et al., 2006). EAE can be actively induced by peripheral immunisation with myelin antigens (or astrocyte and neuronal antigens) (Bernard and Carnegie, 1975; Gold et al., 2006) and can also be adoptively transferred by encephalitogenic $CD4^+$ T cells or $CD8^+$ T cells into a naïve animal (Berger et al., 1997; Huseby et al., 2001; Schluesener and Wekerle, 1985). It has also been spontaneously induced in transgenic mice expressing myelin-specific T cell receptors (TCRs) (Bettelli et al., 2003; Waldner et al., 2000). Theiler's virus encephalomyelitis is an alternative model of MS based on chronic virus induced inflammation and demyelination (Rodriguez et al., 1983).

In actively induced EAE, mice are immunised with peptide epitopes of myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocytes glycoprotein (MOG). These encephalitogenic antigens are emulsified with CFA in order to enhance the immune response to immunisation and to obtain a higher disease incidence (Bernard and Carnegie, 1975; Gold et al., 2006; Mendel et al., 1995; Trotter et al., 1987). Intravenous injections of pertussis toxin (PT) are also included in some protocols to facilitate the induction of EAE (Munoz and Mackay, 1984). PT promotes EAE by increasing permeability across the blood-brain barrier (BBB) and facilitating the entrance of pathogenic T cells to the CNS, in addition to stimulating innate immune responses through Toll like receptor (TLR) 4 (Kerfoot et al., 2004). Around two weeks post-immunisation mice develop encephalomyelitis, characterised by microglial activation, perivascular infiltrates of lymphocytes and macrophages in the CNS white matter and anti-myelin antibodies (only in some mice strains and after immunisation with MOG), which is all followed by demyelination. Inflammation in

EAE usually starts in the lumbar region of the spinal cord, spreading to the entire spinal cord by the peak of disease, which is reflected as transient ascending hind-limb paralysis (Brown et al., 1982; Gold et al., 2006). EAE is a T-cell dependent disease, mediated by $CD4^+$ T helper 1 (T_H1) and T helper 17 (T_H17) cells that produce pro-inflammatory cytokines such as interferon (IFN) γ and IL-17. Anti-myelin T_H1 and T_H17 cells migrate into the CNS where they get re-activated by resident antigen presenting cells (APCs) and secrete soluble mediators that induce inflammation by recruiting monocytes and more T cells to the CNS parenchyma and by activating microglia (Ando et al., 1989; Komiyama et al., 2006; Park et al., 2005; Powell et al., 1990).

The murine models of EAE include acute, chronic, and relapsing-remitting disease, which is mainly dependent on the immunizing antigen, the mouse strain used and its genetic background (Gold et al., 2006). MBP-induced EAE in the PL/J strain is usually characterised by an acute paralytic episode from which the mice recover either partially or totally (Zamvil et al., 1988), while EAE in C57BL/6 mice immunised with MOG or MOG_{35-55} is usually observed as a chronic monophasic disease with partial recovery (Mendel et al., 1995). Chronic relapsing-remitting EAE (CR-EAE) is normally seen in $PLP_{139–151}$ immunised SJL/J mice (Tuohy and Thomas, 1995) and in MOG_{8-21} and MOG_{35-55} immunised Biozzi ABH mice (Baker et al., 1990). In this last model acute disease is accompanied by little demyelination, while marked inflammation and demyelination as well as anti-MOG antibodies are present in the CNS during the relapse phase (Morris-Downes et al., 2002).

The CR-EAE histopathological and clinical similarities with relapsing-remitting MS, makes it an ideal model to study the molecular basis of both disease induction and progression (Amor et al., 2005).

1.1.4. Immunopathogenesis of MS

The immunopathogenesis of MS has not been completely elucidated, but the disease is considered to be caused by an abnormal immune response against myelin self antigens, initiated by autoreactive T cells. The heterogeneity of MS clinical presentation, severity and pathological characteristics of lesions suggest the involvement of multiple mechanisms in the disease pathogenesis (Frohman et al., 2006; Lucchinetti et al., 2004).

The myelin sheath surrounds axons in the white matter of the brain and spinal cord, providing throphic support, protection and insulation that facilitate conduction of nerve impulses (Wilkins et al., 2003). Axons are slim, long neural projections that conduct sensory stimuli and motor responses to and from the neuron's cell body in the form of electrical impulses. Myelin is made of elongated oligodendrocytes' processes that encircle an axon and extend along it to form a myelinated segment. Ranvier nodes are located in between the axonal myelinated segments and contain sodium channels. Electrical resistance is low in these nodes, facilitating the production of action potentials and the production of electrical current that "jump" from one node to another (saltatory conduction), resulting in a faster propagation speed (Compston and McAlpine, 2005).

The events that lead to the development of MS are believed to start with the activation of autoreactive $CD4^+$ T cells in the periphery, which transform them into effector T_H1 and T_H17 cells and upregulate surface cell adhesion molecules (like integrin VLA-4) (Kebir et al., 2007; Sospedra and Martin, 2005; Steinman et al., 2002). The mechanisms by which autoreactive T cells are activated in the periphery are unclear. However, this activation could occur following the recognition of viral peptides by molecular mimicry (McCoy et al., 2006) or through the presentation of CNS antigens by APCs in deep cervical lymph nodes (Flugel et al., 2001), on the background of genetic susceptibility. Tumor necrosis factor (TNF) α and IFNγ produced during a systemic infection might activate the perivascular endothelial cells of the blood-brain

barrier (BBB) and up-regulate the expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) (Doukas and Pober, 1990; Kerfoot et al., 2004; Sospedra and Martin, 2005), which allow the activated autoreactive T cells to transmigrate into the CNS. Alternatively, T cells that enter the sub-arachnoid space (SAS) at the choroid plexus (where adhesion molecules are constitutively expressed) as part of normal immune surveillance might get activated by SAS macrophages presenting CNS antigens, triggering a pathological pro-inflammatory immune response and activating perivascular endothelial cells (Goverman, 2009).

Once inside the CNS parenchyma, the autoreactive effector $CD4^+$ T cells encounter their self myelin antigens (or other non myelin antigens) and are stimulated to secrete pro-inflammatory cytokines such as IFNγ and TNFα (Olsson et al., 1990; Powell et al., 1990). These cytokines activate resident microglia and CNS macrophages, which in turn release more pro-inflammatory cytokines (IL-12, IL-6, IL-23, TNFα, IL-1β, etc.) and chemokines (CCL2, CCL3, CCL5, CXCL10, etc.) that recruit and activate other immune cells from the periphery, including monocytes, $CD8^+$ T cells, B cells and more $CD4^+$ T cells, which amplify the autoimmune response (Ajami et al., 2011; Heppner et al., 2005; Kebir et al., 2007; Marik et al., 2007; Raivich and Banati, 2004; Sospedra and Martin, 2005). Matrix metalloproteinases produced by the activated autoreactive T cells and microglia further facilitate this peripheral leukocyte infiltration by disrupting the BBB (Lindberg et al., 2001; Maeda and Sobel, 1996; Marik et al., 2007). This BBB disruption also allows circulating anti-myelin antibodies and complement components to enter the CNS (Lucchinetti et al., 2000; Storch et al., 1998). Additionally, cytokines produced by the effector T cells and activated macrophages and microglia, such as IFNγ, IL-23 and IL-6, promote pro-inflammatory T_H1 and T_H17 differentiation of newly infiltrating naïve $CD4^+$ T cells (Bettelli et al., 2006; Mosmann et al., 1986; Park et al., 2005). Activated CNS macrophages, microglia and infiltrating macrophages become APCs that activate these later infiltrating T cells, resulting in a further amplification of

the inflammatory response accompanied by T cell proliferation and production of added pro-inflammatory mediators and toxic substances that cause tissue damage (Greter et al., 2005; Hickey and Kimura, 1988; Juedes and Ruddle, 2001; Mack et al., 2003).

Numerous mechanisms might bring about the immune-mediated injury of the myelin sheath, oligodendrocytes and axons. These include direct injury by nitric oxide (NO), reactive oxygen species (ROS) and cytokines (TNFα and IFNγ) (Felts et al., 2005; Marik et al., 2007; Smith et al., 2001); antibody-mediated complement activation and cytolis; antibody-dependent myelin phagocytosis (Berger et al., 1997; Linington et al., 1988; Storch et al., 1998); and direct injury of axons and oligodendrocytes by $CD8^+$ cytotoxic T cells (Bitsch et al., 2000; Medana et al., 2001; Sun et al., 2001) .

The damage to oligodendrocytes and myelin results in denuded axons that are no longer able to transmit action potentials efficiently (Smith et al., 1981; Waxman and Brill, 1978). This slowing or blocking of the axonal conductivity within the CNS causes the signs and symptoms observed during the relapses in the early phase of MS (Compston and Coles, 2008; Gronseth and Ashman, 2000; Youl et al., 1991). When the inflammation resolves, axons are partially re-myelinated by surviving oligodendrocytes or by the recruitment and differentiation of resident oligodendrocyte precursor cells, leading to the functional recovery seen during remissions (Kornek et al., 2000; Patrikios et al., 2006; Scolding et al., 1998; Youl et al., 1991). However, the original thickness of the myelin sheath is not regained and the conduction velocity is slower in these repaired areas (Patrikios et al., 2006). Eventually repeated cycles of demyelination and remyelination exhaust these tissue repair mechanisms (Wolswijk, 1998). Demyelination is also accompanied by compensatory spread of sodium channels from the nodes of Ranvier to the denuded axon segments, which helps to partially restore conduction but is also detrimental. The increased entry of sodium actually slows or even blocks conduction and propitiates a reversal of the sodium-calcium exchanger causing increased

entrance of calcium to the axon. This is followed by calcium-dependent protease mediated injury with a blockade of axonal transport and finally axonal disruption (transection) (Coman et al., 2006; Craner et al., 2004a; Craner et al., 2004b; Howell et al., 2006).

Although axons seem to be relatively spared in acute lesions, axonal injury starts early in the disease course (Bitsch et al., 2000; Ferguson et al., 1997; Kornek et al., 2000). The exposed axon segments lose the trophic support provided by oligodendrocytes (Wilkins et al., 2003) and are susceptible to be damaged by inflammatory soluble mediators (cytokines, NO, ROS, complement, and proteases), which can result in irreversible acute axonal injury (such as axonal transection) (Bitsch et al., 2000; Ferguson et al., 1997; Smith et al., 2001). After repeated relapses, axonal damage and loss accumulate, resulting in permanent and progressive neurologic disability (Bjartmar et al., 2000). The pathological characteristics of the disease also change with time as acute inflammation produced by infiltrating peripheral leukocytes is replaced by mild, but generalised inflammation caused by widespread microglial activation (Kutzelnigg et al., 2005).

1.1.5. Role of the adaptive immune system in MS

1.1.5.1. T cells

EAE studies have demonstrated that autoreactive $CD4^+$ T and $CD8^+$ T cells can cause inflammatory demyelination of the CNS similar to that observed in MS (Ando et al., 1989; Berger et al., 1997; Huseby et al., 2001). Interestingly, MS patients and healthy controls seem to have similar numbers of circulating autoreactive T cells (Burns et al., 1983; Ota et al., 1990). However, myelin-reactive T cells from patients with MS have an activated or memory phenotype, whereas they show a naïve phenotype in healthy people (Lovett-Racke et al., 1998). After antigen encounter, MS myelin-reactive $CD4^+$ T cells tend to adopt a pro-inflammatory T_H1 profile while myelin-reactive $CD4^+$ T cells from healthy donors are more

likely to produce anti-inflammatory T_H2 responses (Crawford et al., 2004). Importantly, $CD4^+$ T cells reactive to myelin antigens tend to be restricted by MS risk associated HLA class II molecules in patients with MS. This has been proven by experiments with humanised transgenic mouse models, where mice expressing an MS patient derived T cell receptor (TCR) specific for an MBP epitope along with HLA-DRB1*1501 and HLA-DRB5*0101 developed spontaneous EAE (Madsen et al., 1999). Additionally, $CD4^+$ T cells from patients with multiple sclerosis have a higher expression of β-arrestin 1, a positive regulator of $CD4^+$ T cell survival that reduces apoptosis (Shi et al., 2007).

For a long time EAE and MS were considered to be mainly mediated by IFNγ producing T_H1 autoimmune responses (Ando et al., 1989). Later EAE studies showed that IL-23 plays a more essential role in CNS inflammation than the closely related IL-12 (Cua et al., 2003). IL-23 along with IL-6 and a few other cytokines produced by microglia and infiltrating macrophages induce T_H17 differentiation of $CD4^+$ T cells and the production of IL-17 (Bettelli et al., 2006; Park et al., 2005). These T_H17 cells are critical for induction of disease activity, at least in the EAE model (Komiyama et al., 2006; Park et al., 2005). The pro-inflammatory cytokine IL-17 might also be important for MS immunopathogenesis, since gene expression and immunohistochemical studies have demonstrated an increased expression of IL-17 as well as an enrichment in MS lesions of both $IL\text{-}17^+$ $CD4^+$ and $CD8^+$ T cells (Lock et al., 2002; Tzartos et al., 2008). Moreover, myeloid dendritic cells of MS patients produce more IL-23 than healthy controls and the cytokine is expressed in both active and chronic MS lesions.

Several studies have shown that lymphocytes other than $CD4^+$ T cells also contribute to the inflammatory response in MS. Autoreactive, neuroantigen-specific $CD8^+$T cells seem to have a higher prevalence in MS patients than in healthy controls (Crawford et al., 2004). In MS lesions $CD8^+$ T cells outnumber $CD4^+$ T cells and they can be found at the edge of the lesions as well as in the perivascular cuffs, while $CD4^+$ T cells are usually only present in the edge (Booss

et al., 1983; Gay et al., 1997). Additionally, the axonal and tissue damage correlates better with the number of infiltrating $CD8^+$ T cells and macrophages than with CD4+ T cells (Bitsch et al., 2000; Booss et al., 1983). CD8+ cytotoxic T cells directly injure oligodendrocytes and axons that express MHC class I via Fas/Fas ligand mediated cytolysis, through contact-mediated lysis via perforin or by directed secretion of cytotoxic granules containing granzyme (Friese and Fugger, 2005).

There is a deficiency of $CD4^+CD25^+$ and $CD8^+$ regulatory T cells in MS patients, which could play a role in the breakdown of tolerance to self-antigens (Balashov et al., 1995). It has also been described that peripheral blood $CD4^+CD25^{hi}$ regulatory T cells from patients with MS show a significant decrease in the effector function compared to healthy donors (Viglietta et al., 2004).

1.1.5.2. B cells

Antibodies against myelin oligodendrocytes glycoprotein (MOG) and myelin basic protein (MBP) have been found in the CNS of patients with MS (Belogurov et al., 2008; Berger et al., 2003). Deposits of immunoglobulins and activated complement can be seen in MS and EAE demyelinated acute lesions (Lucchinetti et al., 2000; Storch et al., 1998). A characteristic clinical finding in MS is the presence of intrathecally synthesised immunoglobulin G in the cerebrospinal fluid of patients. This is visualised as oligoclonal bands on gel electrophoresis and reflects the presence of antigen specific B cells in the CNS (Colombo et al., 2000), usually in meningeal follicules (Magliozzi et al., 2007).

1.1.6. Role of the CNS innate immune system in MS

The innate immune system has an important role in the initiation and progression of MS both by influencing the effector function of T cells and by the direct production of tissue damage through innate immunity effector mechanisms (Sospedra and Martin, 2005). This is evidenced by the presence of infiltrating monocytes and macrophages as well as activated microglia in the inflammatory CNS lesions of MS patients (Lucchinetti et al., 2000; Marik et al., 2007).

1.1.6.1. Microglia

Microglia are the myeloid resident innate immune cells of the CNS (del Rio-Hortega, 1932). Given the obvious difficulties of studying human microglia *in vivo*, most of what is known about these cells under physiological and pathological conditions comes from studies carried out in mouse models and cultured cells. *In vivo* lineage tracing studies in mice have concluded that adult microglia derive from primitive myeloid progenitors originating from the yolk sac before embryonic day 8, which then migrate into the brain through blood vessels between embryonic day 8.5 and 9.5 (before the start of definitive haematopoiesis in the liver, spleen and the bone marrow) (Ginhoux et al., 2010). Hence microglia are part of the mononuclear phagocyte system but constitute a distinct population, since tissue macrophages derive from bone marrow haematopoietic stem cells (van Furth and Cohn, 1968). Furthermore, parabiosis studies (a technique that surgically connects the circulatory system of two animals) in mice have shown that in the healthy adult CNS, circulating myeloid progenitor cells or monocytes do not contribute to the maintenance of the microglial population. Instead the microglial population has very little turn-over and is sustained by local self renewal (Ajami et al., 2007; Ginhoux et al., 2010).

Under physiological conditions microglia show a ramified morphology and are in a relatively latent state, although they constantly survey their local environment through their processes

(Nimmerjahn et al., 2005). This repressed, surveying phenotype is mainly sustained by inhibitory interactions with neurons. CX3CL1, a chemokine produced by neuronal cells, binds to the cell surface receptor CX3CR1 and restrains microglial activation. In CX3CR1 knockout mice, Parkinson's disease and amyotrophic lateral sclerosis models, microglia become hyper-reactive resulting in exaggerated neuronal loss after peripheral lipopolysaccharide (LPS) injections (Cardona et al., 2006b).

Neurons also control microglia via cell contact mechanisms. The cell surface neuronal proteins CD200, CD47, CD22 and HSP60 interact with the microglial receptors CD200R, CD172, CD45 and TREM2 respectively, providing calming signals to microglia (Hoek et al., 2000; Junker et al., 2009; Mott et al., 2004; Neumann and Takahashi, 2007; Piccio et al., 2007).

Microglia can adopt a range of different activation states depending on the type and intensity of the stimulus and the surrounding environment. The outcomes of these states may be neuroprotective, repairing, phagocytic or pro-inflammatory. The microglial activation phenotype may also change throughout a pathological process by adapting to the signals received from other CNS cells and infiltrating leukocytes (Block et al., 2007; Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

Microglia constitute a different cell population from peripheral macrophages. Therefore, the concepts of classical or M1 activation (activation by IFNγ and TNFα produced by T_H1 lymphocytes that results in NO and TNFα production) and alternative or M2 activation (activation in response to IL-4 or IL-13 produced by T_H2 lymphocytes and granulocytes with an important role in responses to parasite infection) that have been used to classify macrophages (Mosser and Edwards, 2008) are not directly applicable to microglia. However, in response to big insults such as infections, CNS tissue injury or inflammation, microglial cells rapidly become activated and change to amoeboid microglia (Kreutzberg, 1996), adopting a phenotype that resembles that of M1 activated macrophages. *In vitro*, this type of pro-

inflammatory activated microglia (or reactive microglia) release a variety of soluble factors, including ROS, NO, cytokines (IL-1β, IL-6, IL-12, TNFα, etc.) and chemokines (CCL3, CCL5, CXCL10, etc.) (Block et al., 2007; Lee et al., 1993; Olson and Miller, 2004). They also increase the expression of surface molecules involved in antigen presentation such as MHC class II, CD80, CD86 and CD40 (Carson et al., 1998; Olson and Miller, 2004). Moreover, reactive microglia secret IL-12 which promotes differentiation of $CD4^+$ T cells into T_H1 cells, as well as IL-23, transforming growth factor-β (TGF-β), IL-6 and IL-1β that induce $CD4^+$T cell differentiation into activated T_H17 cells (Li et al., 2007; O'Keefe et al., 2002; Olson and Miller, 2004). Therefore activated microglia are not only capable of starting innate immune responses, but could also contribute to the development of adaptive immune responses in the CNS. Additionally, activated microglia can proliferate, a process that under pathological conditions is known as reactive microgliosis (Block et al., 2007).

Microglia can become over-activated and start an exaggerated immune response against pathogens or misinterpret non-pathogenic stimuli as harmful. Such stimuli can include environmental toxins and the products of neuronal and glial cell damage or death caused by neurodegenerative disease, ischemia and brain trauma. Over-activated microglia produce exaggerated amounts of inflammatory mediators causing further cellular death and amplification of ongoing tissue damage that exacerbates disease processes (Block et al., 2007; Hanisch and Kettenmann, 2007). Several endogenous ligands designated as damage associated molecular pattern molecules (DAMPs) are released by injured or necrotic cells and these can be detected by scavenger receptors, toll like receptors, receptors for advance glycation end products (RAGE), integrins and metabotropic purine receptors present in the surface of microglia. Such DAMPs include high mobility group box 1 protein (HMGB1), heat shock proteins, histones, oxidised lipids, DNA and ATP among others. In addition, proteases

and hydrolases released by dead cells degrade the extracellular matrix generating more DAMPs, such as fibrinogen, heparan sulphate and hyaluronic acid (Kono and Rock, 2008).

In MS, microglial cells can become activated through different mechanisms. These include activation by the pro-inflammatory cytokines released from T and B cells, activation secondary to the phagocytosis of myelin and myelin debris, and activation via TLR receptors, probably by pathogens or endogenous ligands (Sriram, 2011). In active MS and EAE lesions, over-activated microglia release ROS, NO and pro-inflammatory cytokines and chemokines that cause extensive damage to neurons, axons, oligodendrocytes and myelin (Bjartmar et al., 2001; Raivich and Banati, 2004; Rasmussen et al., 2007). Furthermore, activation of resident innate immune cells within the CNS has been demonstrated to be vital for the development and maintenance of inflammatory autoimmune responses (Ajami et al., 2011; Heppner et al., 2005; Marik et al., 2007).

Whether or not microglia contribute to the initiation and progression of MS and EAE as one of the APCs that instigate the autoimmune adaptive response is still in debate. It has been shown through immunohistochemistry that reactive microglia in MS and EAE upregulate MHC class I and II as well as the costimulatory molecules CD40, CD80 and CD86 (Hayes et al., 1987; Ponomarev et al., 2005; Windhagen et al., 1995), which in theory would enable them to present antigens to T cells. EAE studies in which microglia and macrophages have been isolated from the diseased CNS by flow cytometric sorting have yielded contradictory results. Some reports have shown that macrophages, but not microglia, are the effective APCs in EAE, while others have reported that microglia, as well as macrophages, are able to stimulate proliferation and IFN-γ production in T cells (Ford et al., 1995; Juedes and Ruddle, 2001; Mack et al., 2003). However, one caveat of these studies is that they have relied on CD45 (a cell surface tyrosine phosphatase present on all hematopoietic cells) expression to differentiate microglia from macrophages as first proposed by Sedgwick *et al*. $CD45^{low}$ cells were

considered as the resident microglia and $CD45^{high}$ cells as the CNS and peripheral macrophages (Sedgwick et al., 1991). However, it has been shown that reactive microglia also up-regulate CD45 along with other morphology changes that render them indistinguishable from macrophages (Ponomarev et al., 2005; Sedgwick et al., 1991). Therefore some of the cells labelled as macrophages in these studies might actually have been activated microglia. Despite this, it seems that blood-derived APCs located in the perivascular space are the main cells that present antigen to T cells, at least in the case of EAE (Greter et al., 2005; Hickey and Kimura, 1988).

Two EAE studies have shed light on the distinct roles of these two near morphologically identical populations in the disease pathology. In order to distinguish between resident activated microglial cells and infiltrating macrophages, Ponomarev *et al.* employed MHC mismatched donor-recipient bone marrow chimera mice (Ponomarev et al., 2005) while Ajami *et al.* used a combination of green fluorescence protein (GFP)-positive and GFP-negative mice parabiosis and myeloablation (Ajami et al., 2011). The two groups found that microglia become activated early in EAE disease (or even before the onset of symptoms in Ponomarev's study), prior to infiltration of peripheral monocytes into the CNS. This early microglial activation coincided with T cell infiltration. This suggests microglia play a central role in disease initiation, possibly by supporting the differentiation of naïve or myelin-specific $CD4^+$ T cells towards the T_H1 and T_H17 type by producing IL-12 and IL-23, rather than by presenting antigens. Additionally, such activated microglia multiplied extensively, as measured by bromodeoxyuridine incorporation into cellular DNA (Ajami et al., 2011; Ponomarev et al., 2005). Ajami *et al*. showed that when peripheral monocytes could not reach the CNS, mice developed mild EAE that did not progress to paralysis, demonstrating that whilst resident microglia and T cells are responsible for disease initiation, disease progression is dependent on temporary monocyte infiltration (Ajami et al., 2011). Infiltrating

macrophages were phenotypically indistinguishable from activated microglia in the CNS parenchyma. Moreover, at the peak of EAE disease, nearly 40% of the cells considered as macrophages ($CD45^{high}$ cells) were actually activated microglial cells and co-localised with the infiltrating macrophages in inflammatory lesions (Ajami et al., 2011; Ponomarev et al., 2005). After EAE recovery, no remaining infiltrating macrophages were detectable in the CNS and whilst most microglia underwent apoptosis at the peak of disease, proliferated microglia were present a long time after disease remission (Ajami et al., 2011).

Interestingly, microglia outside the inflammatory lesions, in the normal-appearing white matter (NAWM) and the gray matter (cortex), also show an activated phenotype in MS and EAE (Banati et al., 2000; Bjartmar et al., 2001; Rasmussen et al., 2007; Zeis et al., 2008). In a study with a relapsing-remitting EAE model in SJL mice that develop forebrain lesions that resemble those observed in MS, microglia acquired an activated morphology (enlarged cell body with short, tick processes) at the onset of disease that reverted during the first remission. However, after the first relapse, microglia remained permanently activated even during remissions, in which infiltrating T cells have left the CNS, and did not return to the surveying ("resting") morphology that they exhibit in the normal CNS (Rasmussen et al., 2007). Such chronic microglial activation has also been described in MS tissue during the progressive stage of disease, in which activated microglia are present throughout the whole NAWM and correlate with neuronal dysfunction, loss of neuronal synapses and axonal degeneration. Therefore, chronically activated microglia contribute to the permanent neurological disability observed in progressive patients by causing widespread axonal and neuronal injury, which is independent of the initial auto-inflammatory response (Kutzelnigg et al., 2005).

1.1.6.2. TLRs and activation of inflammation

The TLRs are pattern recognition receptors employed by the innate immune system to recognise pathogen-associated molecular patterns (PAMPs) and initiate the appropriate protective immune response against microorganisms, including bacteria, viruses, protozoa and fungi (Medzhitov and Janeway, 1998). The TLRs can be divided in two groups according to their cellular localisation. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface and mainly identify lipids, lipoproteins and protein parts of microbial membranes. TLR3, TLR7, TLR8 and TLR9 are localised in intracellular vesicles and recognise pathogen nucleic acids. Each TLR recognises one or more specific conserved pathogen structures and, following ligand binding, form homodimers, with the exception of TLR2, which makes heterodimers with TLR1 and TLR6 (Akira et al., 2006). TLR1/2 and TLR2/6 recognise lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from Trypanosoma cruzi and the hemaglutinin protein from measles virus. Specifically, TLR1/2 recognises triacylated lipopeptides from Gram-negative bacteria and mycoplasma while TLR2/6 recognises diacylated lipopeptides from Grampositive bacteria and mycoplasma (Akira et al., 2006; Takeuchi et al., 1999). TLR3 identifies double stranded viral RNA (Alexopoulou et al., 2001). TLR4 in complex with MD-2 binds to LPS, a component of the outer membrane of Gram-negative bacteria (Poltorak et al., 1998). TLR5 recognises the bacterial protein flagellin (Hayashi et al., 2001), TLR7 and TLR8 single-stranded viral RNA (Heil et al., 2004), and TLR9 unmethylated cytosine-guanosine (CpG) DNA frequently present in bacteria and viruses (Hemmi et al., 2000).

TLRs are type I integral membrane glycoproteins with extracellular domains containing leucine-rich-repeat (LRR) motifs that mediate ligand binding and cytoplasmic signal transduction Toll–interleukin 1 receptor (TIR) domains (Bowie and O'Neill, 2000). Upon

ligand binding, TLR dimmers recruit TIR domain containing adaptor proteins to their cytoplasmic portion which activates downstream signalling cascades. The signalling pathways activated by TLRs are classified into myeloid differentiation factor 88 (MyD88) dependent pathways and TIR-domain-containing adaptor-inducing interferon-β (TRIF) dependent pathways (Akira et al., 2001). All the TLRs, with exception of TLR3, signal through the MyD88 dependent pathway which ultimately leads to the activation of the transcription factors NF-kB and AP-1 and production of inflammatory cytokines, such as IL-1, IL-6, IL-12 and TNFα, chemokines and induction of costimulatory molecules (Medzhitov et al., 1998). TLR3 and TLR4 use the TRIF-dependent pathway that culminates in the activation of interferon regulatory factor 3 (IRF3) and NF-κB and induction of type I interferon as well as inflammatory cytokines (Yamamoto et al., 2003).

TLRs can also be activated by endogenous ligands, initiating inflammatory responses without the presence of pathogens (Zhang and Schluesener, 2006). Several TLR 2 and 4 endogenous ligands have been reported including products from the breakdown of the extracellular matrix by cellular proteases released from dying cells. These include: hyaluronate, heparan sulphate, biglycan, and extradomain A of fibronectin (Gondokaryono et al., 2007; Johnson et al., 2004; Taylor et al., 2004). Other intracellular molecules released by damaged or necrotic cells include the nuclear high mobility group box 1 protein (HMGB1) and heat shock proteins. These intracellular molecules, in addition to plasma fibrinogen leaked from the blood vessels to the intercellular space during inflammation, are all TLR2 and TLR4 ligands (Ohashi et al., 2000; Park et al., 2004; Smiley et al., 2001). TLR 3, 7 and 9 can become activated by self-nucleic acids released from dead or dying cells that avoid cleavage by serum nucleases and get inside the cell by forming complexes with endogenous proteins such as ribonucleoproteins and antibodies, promoting systemic autoimmune diseases (Boulé et al., 2004; Brentano et al., 2005; Vollmer et al., 2005). Some endogenous TLR ligands have been found in MS lesions.

HMGB1, a pro-inflammatory TLR2 and TLR4 endogenous ligand released by necrotic cells but also from stimulated monocytes and macrophages, is highly expressed by microglia and macrophages in active MS and EAE lesions and its levels correlate with inflammation (Andersson et al., 2008). The coagulation cascade plasma protein fibrinogen, and its active form fibrin, are also endogenous TLR4 ligands that have been found in acute MS lesions secondary to blood brain barrier leakage (Gay and Esiri, 1991). Early in MS lesions precipitated fibrin can be seen in the surface of activated microglia, suggesting a causal relationship with activation (Marik et al., 2007). Fibrin has also been shown to increase neurodegeneration in EAE (Adams et al., 2007). Oxidised lipids, by-products of the free oxygen radicals mediated oxidation and breakdown of myelin, are present in the cerebrospinal fluid (CSF) and serum of MS patients and can activate microglia via the TLR2 receptor (Diestel et al., 2003; Farez et al., 2010). Additionally, it is reasonable to hypothesise that other endogenous ligands which are products of inflammation-related tissue injury might also be present in the CNS of MS patients. For example, heat shock proteins 60 (HSP60) released from necrotic or apoptotic CNS cells activates microglia through TLR4, inducing the release of NO and widespread neuronal death as well as axonal loss in murine primary CNS cultures (Lehnardt et al., 2008).

Human and murine microglia express TLRs 1 to 9 (Bsibsi et al., 2002; Jack et al., 2005; Olson and Miller, 2004). Various studies with human and murine cultured microglia, as well as with mouse models, have shown that TLR2, 3, 4 and 9 stimulation in “resting” or surveying microglia contributes to both innate and adaptive immune responses, leading to the production of inflammatory mediators (IL-1β, IL-6, IL-10, IL-12, TNFα, CCL3, CCL5, CXCL10 and NO) and up-regulation of APC surface molecules as well as the TLRs expression (Becher and Antel, 1996; Dalpke et al., 2002; Ebert et al., 2005; Jack et al., 2005; Kielian et al., 2005; Lehnardt et al., 2003; Olson and Miller, 2004; Town et al., 2006).

TLR activation of microglia in MS and EAE most probably plays a role in the adaptive autoimmune response initiation and development through different mechanisms. Firstly TLR activation induces microglial production of chemokines such as CCL2, CCL5 and CXCL10, which recruit and facilitate the infiltration of peripheral monocytes and auto-reactive T cells to the CNS (Jack et al., 2005; Olson and Miller, 2004). Secondly, microglia stimulated by TLR agonists secrete cytokines that promote T_H1 and T_H17-type pro-inflammatory responses (O'Keefe et al., 2002; Olson and Miller, 2004). Lastly, microglial activation via TLR agonists up-regulates the expression of costimulatory molecules and MHC class II, which could enable the cells to present myelin antigens to auto-reactive T cells (Becher and Antel, 1996; Dalpke et al., 2002; Olson and Miller, 2004). Regardless of the mechanism of adaptive immune response, the innate component of TLR microglial activation also contributes to the MS and EAE pathophysiology via the release of cytotoxic and pro-inflammatory substances (Brenner et al., 1997; Marik et al., 2007). In mixed glial cultures, activation of microglia with the TLR4 agonist LPS damages and kills both oligodendrocytes and neurons (Lehnardt et al., 2003). Likewise, intracerebral injection of LPS in neonatal rats produces oligodendrocyte death and hypomyelination, (Lehnardt et al., 2002; Pang et al., 2003). It has also been described that infections can exacerbate and even initiate MS (Buljevac et al., 2002; Kurtzke, 1993). Hence, TLR activation of microglia by either PAMPs or DAMPs plays an important role in the induction and regulation of MS disease.

In MS active lesions there is a high expression of TLR3 and TLR4 in microglia, particularly in perivascular areas at the centre and the borders of the lesions (Bsibsi et al., 2002). In EAE, an increase in TLR1, TLR2, TLR4, TLR6, TLR7, TLR8 and TLR9 as well as MyD88 within the spinal cord has been shown, with TLR9 being increased later in the disease than the other TLRs (Prinz et al., 2006). The results obtained by diverse studies on the influence of different TLRs in EAE initiation and progression are contradictory. This has been attributed to the use of

different EAE induction protocols. However, MyD88 has consistently been shown to be necessary for EAE induction (Marta et al., 2008; Prinz et al., 2006), suggesting that TLR signalling through this adaptor plays an essential role in EAE and perhaps in the development of autoimmune CNS disease. Lethally irradiated MyD88$^{-/-}$ mice reconstituted with wild type (WT) bone marrow, in which microglia remain MyD88$^{-/-}$, continue to be susceptible to EAE induction but show a much milder disease course. This implicates microglial TLR signalling as an important player in the disease. Furthermore it seems that poor T cell priming is the cause of the MyD88$^{-/-}$ mice resistance to EAE in this model (Prinz et al., 2006).

Although CFA (the adjuvant used in classical EAE induction) contains mycobacterial PAMPs that bind to TLR2/6 and TRLR2/1, it has been shown these receptors have a redundant function in EAE because TLR2 knockout mice display the same disease course as WT mice (Marta et al., 2008; Prinz et al., 2006). This also suggests that TLR4 or other innate receptors mediate the CFA adjuvant effect. However, a later study has demonstrated that TLR2 binding by 15α-hydroxicholestene (15-HC), an oxidised product of cholesterol which is at an increased concentration in the serum of patients with SPMS and in mice with secondary progressive EAE, promotes pro-inflammatory activation of microglia and monocytes through a pathway dependent on poly (ADP-ribose) polymerase I (PARP-1). Importantly, PARP-1 inhibition *in vivo* suppressed microglial activation, reduced the number of CNS infiltrating macrophages and reduced the expression of CCL2, TNFα and nitric oxide synthase 2 (NOS2), all of which stopped the progression of EAE. Thus, TLR2-PARP-1 pathway might be important for the secondary progression of MS (Farez et al., 2010).

TLR3 activation with poly I:C produced less severe disease and suppressed relapses in a $PLP_{139–151}$ EAE model, which was associated with less inflammatory infiltration and demyelination as well as with IFN-β induction (Touil et al., 2006).

Two reports about MOG induced EAE in TLR4$^{-/-}$ mice have produced opposing results. Kerfoot *et al*. showed that disease incidence was lower in TLR4 knockout mice, with no change in time of onset and severity. They also argued that the pertussis toxin function in the initiation of EAE is partially dependent on TLR4 (Kerfoot et al., 2004). On the other hand, Marta *et al*. found that the lack of TLR4 exacerbates EAE, possibly by impairing MOG-specific T_H1 responses which in turn promotes T_H17 cell mediated inflammation (Marta et al., 2008).

Similarly, TLR9 deficiency has been reported to be both detrimental and beneficial in EAE by different groups. As with EAE induced in TLR4$^{-/-}$ mice, the conflicting results could be due to the different protocols used to induce EAE. In the study where TLR4 and TLR9 knockouts were found to worsen EAE, animals were immunised with whole MOG protein. In the contradictory studies just the immune-dominant MOG_{35-55} epitope was used (Marta et al., 2008; Prinz et al., 2006). The contradictory results could be explained by the fact that immunisation with MOG protein induces pathogenic B cell responses and production of auto-antibodies like the ones observed in MS, whereas immunisation with MOG_{35-55} does not induce these effects (Marta et al., 2009). Marta et al. described a more severe EAE with higher disease scores in TLR9$^{-/-}$ mice compared to WT, providing only an increased expression of Il-6 by splenocytes as a possible explanation and without conducting histological studies (Marta et al., 2008). In contrast, Prinz *et al.* showed that TLR9 deficiency caused a significant delay in disease onset as well as lower disease scores. In their study, the TLR9 knockout mice had smaller infiltrates and less demyelination and axonal damage at late stages of EAE than WT mice. This was dependent on TLR9 absence from both peripheral leukocytes and microglia. Interestingly, it appears that an endogenous molecule is responsible for the TLR9 activation in EAE because the presence of possible TLR9 ligands in the adjuvant was ruled out (Prinz et al., 2006).

Overall it seems that TLR4 signalling is mostly involved in the initial phases of EAE, whereas TLR2 and TLR9 are involved in modulating EAE progression and TLR3 activation may be protective. Whether or not these findings are also applicable to MS is not known.

1.1.6.3. CNS macrophages

Apart from microglia, which are by far the most common innate immune cells in the CNS, other members of the mononuclear phagocyte system are also found in the healthy CNS. These macrophage populations include perivascular macrophages (PVMs), meningeal macrophages, and circumventricular organs and choroid plexus macrophages. While microglia are located inside the CNS parenchyma, CNS macrophages are located outside the basal membranes of vessels, meninges, ventricular epithelium and chorioepithelium, and thus are not in contact with neurons and glial cells. These CNS macrophages show significant morphological, biochemical and cell membrane marker differences to microglia. These differences include a higher expression of MHC II (Raivich and Banati, 2004; Ransohoff and Cardona, 2010) and gradual replenishment of populations by circulating monocytes (Hickey and Kimura, 1988; Streit et al., 1989). In MS and EAE, CNS macrophages, as well as the peripheral monocytes located in the perivascular infiltrates, are considered to be the main APCs to auto-reactive T cells (Bartholomaus et al., 2009; Gehrmann et al., 1993; Hickey and Kimura, 1988; Waldner et al., 2004).

1.2. Epigenetics

Epigenetics is the study of stable, heritable changes in the genome that influence gene expression without changing the DNA sequence. The mechanisms of epigenetic gene

regulation include DNA methylation, histone tail modifications and miRNA-mediated post-transcriptional gene silencing (Jaenisch and Bird, 2003; Jirtle and Skinner, 2007).

1.2.1. Transcription regulation and modification of chromatin

In eukaryotic cells transcription initiates with the binding of one or more regulatory proteins (termed transcriptional activators) to specific DNA sequences (enhancers) that can be several kilobases upstream or downstream from the transcription start site (TSS). Despite being far away, activators get access to the promoter region of the genes through DNA looping. Activators then recruit ATP-dependant chromatin remodelling complexes that displace nucleosomes at the promoter, making the DNA available for transcription, as well as large protein co-activator complexes including the SAGA histone acetyltransferase complex and Mediator. These co-activators and nucleosome remodelers facilitate the rapid recruitment of the transcription machinery in a sequential order. First, the general transcription factor TFIID binds to a TATA box (short double-helical DNA sequence composed mainly by T and A nucleotides) or to other initiation of transcription consensus DNA sequences located 25 nucleotides upstream from the transcription start site (TSS) in the promoter. This causes a big distortion in the DNA of the TATA box which allows the binding of TFIIB followed by the other general transcription factors (including TFIIF), as well as the RNA polymerase II (Pol II) (enzyme that transcribes DNA into RNA), assembling the transcription pre-initiation complex. SAGA and Mediator facilitate the interaction of activator proteins with the Pol II and the general transcription factors by providing structural support for the complex to assemble (Casamassimi and Napoli, 2007; Fuda et al., 2009; Grant et al., 1997; Roeder, 2005). Next, the DNA helicase subunit of TFIIH unwinds 11-15 bases of DNA at the transcription start site, exposing the template strand to the active site of the Pol II. Then the protein kinase subunit of TFIIH phosphorylates the carboxy-terminal domain (CTD) of Pol II causing conformational

changes that allow the polymerase to be released from the transcription initiation complex and move away from the promoter (Saunders et al., 2006). Following promoter clearance, the Pol II transcribes short lengths of RNA into the gene body, stopping at the promoter-proximal pause site. In order for the elongation phase of transcription to start, the polymerase CTD requires a second phosphorylation by P-TEFb to create binding sites for proteins important for mRNA processing. The elongating Pol II also requires elongation factors which help the polymerase to stay bound to the DNA it is transcribing and to move along it. Elongation factor proteins include chromatin remodelling complexes and histone modifying enzymes such as the H3K36 methyltransferase SET2 and the H3K4me2/me3 methyltransferase SET1 (Egloff and Murphy, 2008; Fuda et al., 2009; Lee et al., 2007).

One gene can have several activator proteins that often work in complexes. Activators increase the rate of transcription initiation by facilitating the assembly of the transcription pre-initiation complex through the recruitment of chromatin remodelling complexes and histone modifying enzymes that in turn change the chromatin structure at the promoter, making DNA accessible. Histone modifications also allow the binding of other effector proteins for transcription initiation. These include other histone-modifying enzymes, chromatin remodelling complexes and the general transcription factor TFIID (Agalioti et al., 2002; Agalioti et al., 2000; Narlikar et al., 2002; Roeder, 2005).

The order in which the many proteins required for transcription initiation (activators, histone modifying enzymes, chromatin remodelling complexes, SAGA, Mediator, general transcription factors and Pol II) come into play differs from gene to gene, as well as the regulatory proteins needed, as illustrated in figure 1.1. For some inducible genes the activator protein must bind to the chromatin first so the events leading to transcription initiation can start (Roeder, 2005; Weake and Workman, 2010). However, in some other genes, especially in the ones in charge of developmental control, paused Pol II can be found already binding the promoter and poised

for transcription before activators bind regulatory sequences (Boettiger and Levine, 2009; Min et al., 2011).

There are thousands of different regulatory proteins and they include both activators and repressors that bind to DNA regulatory sequences and control the rate of the assembly processes at the promoter allowing genes to be switched on or off individually. Repressors block the assembly of the transcription pre-initiation complex by competing with activators to bind the same regulatory region. They do this by binding to the activators and blocking their activation surface or by directly interacting with the general transcriptional factors and hindering their assembly. Moreover repressors can also recruit chromatin remodelling complexes that pack the DNA back into chromatin making it unavailable for transcription. They also recruit histone modifying enzymes that remove transcription activation marks (histone deacetylases) or deposit repressive marks (some histone methyl transferases) (Roeder, 2005; Weake and Workman, 2010).

Chromatin remodelling proteins, histone modifying enzymes and other proteins that do not bind directly to the DNA, but interact with regulatory proteins favouring or obstructing transcription, are termed co-activators or co-repressors (Qiu et al., 2004; Roeder, 2005; Weake and Workman, 2010).

Modifications that occur during transcription initiation can be rapidly reversed after the activators that set them in place dissociate from DNA. This allows the quick switch on and off of genes in response to external signals. However, under certain circumstances the modifications persist even when the activators have left, and this can be passed to the next cell generation affecting the gene expression patterns in the long term (Strahl and Allis, 2000; Turner, 2007).

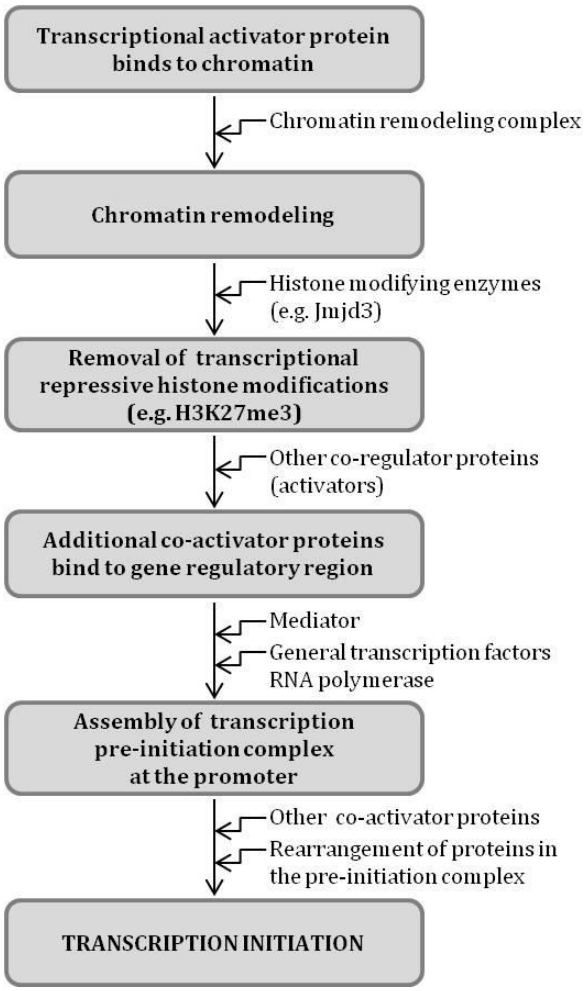


Figure 1.1. Sequence of events leading to transcription initiation. The diagram shows one possible order of the steps towards transcription initiation. This order, as well as the proteins needed for the initiation of transcription, differ from gene to gene (modified from (Alberts, 2008).

1.2.2. Histone modifications

The nucleosome is the basic unit of the chromatin structure, comprising of an octamer of the four core histone proteins (H3, H4, H2A and H2B) around which 147 base pairs (bp) of DNA are coiled in 1.75 turns. Histones have globular bodies and amino-terminal tails that radiate from the nucleosome surface (Luger et al., 1997). These tails can undergo a wide range of post-translational modifications of specific amino acids, including acetylation of lysines,

methylation of lysines and arginines, phosphorylation of serines and threonines, ubiquitination and sumoylation of lysines, ADP ribosylation of glutamic acid, deamination of arginines and proline isomerisation (Kouzarides, 2007).

Histone modifications regulate the transcription of genes by altering the chromatin structure and by the recruitment of non-histone proteins that mediate downstream functions. The modifications can disrupt histone-DNA and histone-histone contacts by neutralising the positive charge of the histone tails, releasing the chromatin and allowing access to the transcriptional machinery. They also create favorable interaction surfaces for effector proteins that contain specialised binding domains, such as histone-modifying enzymes, chromatin remodelling complexes and other co-activators or co-repressors (Agalioti et al., 2002; Jenuwein and Allis, 2001; Margueron et al., 2005; Strahl and Allis, 2000).

In combination, different histone modifications establish a "code" that can be recognised by specific effector proteins and determine whether or not, and the extent to which, genes are expressed (Jenuwein and Allis, 2001). This "crosstalk" can take place between modifications in the same histone (*cis*) or different histones (*trans)* (Nightingale et al.). The epigenetic code regulates the expression of key genes during development and later on maintains the gene expression programs characteristic of differentiated cells (Ringrose and Paro, 2007).

The histone tail modifications are dynamic, being placed and removed by specific modifying enzymes whose activities can be modulated by environmental and intrinsic signals (Turner, 2007). Some of these enzymes can have other non-histone substrates and may accomplish their functions through non-epigenetic mechanisms (Strahl and Allis, 2000).

Histone modifications can have a role in both the short-term ongoing cellular processes (transcription, DNA replication and repair) and in more long-term functions (heterochromatin formation, maintenance of differentiated cells specific gene expression patterns). The levels of short-term modifications fluctuate rapidly, whilst long-term, stable

modifications have slow turnover with a relatively consistent steady-state level (Turner, 2007).

1.2.2.1. Histone lysine methylation

Methylation of histones can occur on arginine and lysine residues. Lysine residues may be mono-, di- or tri-methylated. Lysine methylation does not change the charge of the histone tails and therefore does not disrupt the histone-DNA contacts. The mechanism by which histone methylation affects transcription is by being a binding site for effector proteins. The effector proteins recognise specific methylation marks and thereby the position of the modified residue in the histone, as well as the number of methyl groups added (mono-, di- or trimethylation) (Klose and Zhang, 2007). Methylation at histone 3 lysine 4 (H3K4), histone 3 lysine 36 (H3K36) and histone 3 lysine 79 (H3K79) have been associated with transcriptional activation whilst trimethylation of histone 3 lysine residue 9 or 27 (H3K9me3 and H3K27me3) and histone 4 lysine 20 monomethylation are associated with transcriptional repression (Klose and Zhang, 2007; Kouzarides, 2007). Histone methyltransferases (HMT) belong to the SET-domain containing protein family and are very specific, usually modifying a specific lysine residue within a defined histone (Martin and Zhang, 2005).

Until recently, histone methylation was considered to be a permanent modification since no histone demethylases had been identified (Agger et al., 2008). However, the discovery of histone lysine demethylases suggested that histone methylation could be dynamically regulated. The demethylation of mono- and di-methylated lysines is carried out by amine oxidases, and lysine tri-methylation is removed by 2-oxoglutarate oxygenases by hydroxylation (Bannister et al., 2002). This last group includes around 30 enzymes that contain a Jumonji C (JmjC) domain which catalyse lysine demethylation through an oxidative reaction that requires iron [Fe (II)] and α-ketoglutarate (αKG) as cofactors. The methyl group

is hydroxylated and released as formaldehyde (Tsukada et al., 2006). The JmjC-domain-containing proteins catalyse the demethylation of all three lysine methylation states and display high specificity for target residues and the levels of methylation (Klose and Zhang, 2007; Shi et al., 2007). Based on their phylogeny and their domain composition, these enzymes are divided into seven evolutionary conserved subfamilies: JHDM1, JHDM2, JHDM3/JMJD2, PH2/PHF8, JARID1/JARID2, UTX/UTY and JmjC-domain-only family (Klose et al., 2006).

1.2.2.1.1. Histone 3 lysine 27 tri-methylation (H3K27me3)

H3K27me3 is an epigenetic mark for repression of transcription and is important for the maintenance of pluripotency in embryonic stem (ES) cells. Within embryonic stem cells it is usually found in bivalent domains which also contain activating histone marks (H3K4me3). These bivalent domains silence genes that control lineage fate decisions while keeping the genes poised for activation. During differentiation H3K27me3 is down-regulated by demethylases which allows the expression of cell type-specific genes (Surani et al., 2007).

The tri-methylation of H3K27 is catalysed by the histone methyltransferase Ezh2 that, together with Suz12, Eed and RbAp48, compose the Polycomb-repressive complex 2 (PRC2). H3K27me3 is in turn recognised by members of the PRC1 complex through a chromodomain, which blocks the access of transcriptional activators and chromatin remodelling factors to DNA. This hinders the initiation of transcription and results in the silencing of target genes that regulate cell differentiation, embryonic development and proliferation (Schwartz and Pirrotta, 2007).

However, it has also been described that the presence of H3K27me3 in gene promoters can be compatible with transcription, indicating that the histone mark might adopt different transcription regulation roles depending on the gene or the cellular type (Young et al., 2011).

The H3K27me3/me2-specific demethylases are transcriptional co-activators that belong to the ubiquitously transcribed tetratricopeptide repeat UTX/UTY family and they include Utx, Uty and Jmjd3 (Agger et al., 2007; Hong et al., 2007; Klose et al., 2006). Utx is required during development for the activation of HOX genes by demethylating H3K27me3 (Agger et al., 2007). In differentiated cells, Utx demethylase activity is low and constitutive, being responsible for maintaining the steady-state levels of H3K27me3. Uty seems to be the Y chromosome equivalent of Utx. Although Utx and Uty are highly homologous and contain the same TPR protein interaction domains repeats in addition to the JmjC domain, Uty has been described to be enzymatically inactive (Hong et al., 2007). Jmjd3 shows important homology with Utx and Uty, particularly in the JmjC domain, but it lacks the TPR domains (Klose et al., 2006). As in Utx, Jmjd3 also possesses H3K27me3 demethylase activity but shows an inducible expression in a tissue-specific way in differentiated cells and in precise developmental stages (De Santa et al., 2007).

1.2.3. Jmjd3 (Kdm6)

1.2.3.1. Roles in development and senesce

Jmjd3 fulfils an important role in development by working as a transcriptional activator during cellular differentiation.

Jmjd3 has been shown to be required for the neural commitment of embryonic stem cells by promoting the expression of Pax6, Nestin and Sox1. In the case of Nestin, Jmjd3 recruitment coincided with loss of H3K27me3 in bivalent domains at the promoter. However, for Pax 6 and Sox1, Jmjd3 occupancy did not cause any changes on H3K27me3 levels, suggesting that Jmjd3 regulates their expression by other means (Burgold et al., 2008). Jmd3 is also necessary for the terminal differentiation of neuronal stem cells in response to retinoic acid by inducing the expression of neuronal genes through H3K27me3 demethylation (Jepsen et al., 2007). At

later stages of the CNS development Jmjd3 is indispensable for the maintenance of the respiratory rhythm generator pacemaker known as the pre-Bötzinger complex (PBC). Maintenance is achieved by upregulating key PBC-specific genes through the demethylation of H3K27me3. In *Jmjd3* knockout mice the lack of this mechanism causes perinatal lethality secondary to respiratory failure (Burgold et al., 2012).

Jmjd3 is also involved in the control of epidermal differentiation. In human primary keratinocytes Jmjd3 promotes the expression of epidermal differentiation genes by both reducing their levels of H3K27me3 and by a non-described demethylase independent mechanism in genes that lack H3K27me3 in their promoter regions (Sen et al., 2008). In *C. elegans* the Jmjd3 homologue is also required for adequate gonadal development (Agger et al., 2007).

Additionally, Jmjd3 has been implicated in playing a part in cellular senescence as a potential tumor suppressor gene. In response to oncongenic stress Jmjd3 is induced and demethylates H3K27me3 at the INK4A–ARF locus. This up-regulates the expression of the tumor suppressor proteins $p16_{INK4A}$ and $p14_{ARF}$/$p19_{ARF}$, which in turn lead to the arrest of the cell cycle and inhibition of proliferation. In various types of cancer the expression of Jmjd3 has been shown to be reduced, probably secondary to allele loss (Agger et al., 2009).

1.2.3.2. Roles in the immune response and inflammation

To date, Jmjd3 is the only histone modifying enzyme that has been described to have a role in the immune response and inflammation. A previous study demonstrated that in primary bone marrow-derived mouse macrophages exposed to inflammatory stimuli such as LPS and IFNγ, *Jmjd3* is quickly and strongly up-regulated through the transcription factor NF-κB. They also showed that this Jmjd3 up-regulation was followed by a slow down-regulation of H3K27me3 at the promoter of the target gene, bone morphogenetic protein 2 (*Bmp-2*), and increased

transcription after Jmjd3 binding (De Santa et al., 2007). It was later described that in such pro-inflammatory activated primary bone marrow derived macrophages Jmjd3 binds to the transcription start sites (TSS) of several genes, including those encoding chemokines (e.g. *Cxcl2, Cxcl11, Ccl5*), cytokines (e.g. *Tnfα, Il6, Il12*) and other immune response and inflammation mediators like *Nos2*. However, only a group of these genes (*Ccl3, Ccl4, Ccl5, Ccl9, Cxcl11, Il12b*, and *Il6*) were shown to be partially dependant on Jmjd3 for expression in *Jmjd3*$^{-/-}$ foetal-liver derived macrophages. The transcriptional effects of Jmjd3 depletion were produced both by a reduction in Pol_II recruitment and in the transcription elongation, and were independent of its H3K27me3 demethylase activity (De Santa et al., 2009). Conversely, in primary IL-4-induced alternative activated macrophages (M2-macrophages), the expression of M2 marker genes is controlled by an IL-4 dependent Jmjd3 up-regulation, which in turn decreases the dymethylation and trimethylation of H3K27 and promotes transcription. These *in vitro* observations were confirmed in M2-peritoneal macrophages recovered from mice injected intraperitoneally with eggs from the parasite *S mansoni*. Compared to non-injected mice, the M2-peritoneal macrophages from injected mice had increased expression of Jmjd3 and reduced levels of H3K27me3 at specific M2 marker gene promoters in association with Jmjd3 recruitment (Ishii et al., 2009). A further report using M2 macrophages from *Jmjd3*$^{-/-}$ bone marrow chimeric mice demonstrated that Jmjd3 in fact secondarily regulates M2 marker genes transcription by controlling the expression of the transcription factor *Ifr4* in a demethylase activity dependent way (Satoh et al., 2010). Similarly, the enzymatic H3K27me3 demethylase activity of Jmjd3 has been reported to be required for the expression of *Ccl3* and *Il6* in the EL4 T cell line and in an *in vitro* endothelial cell model of ischemia/reperfusion injury, respectively (Lee et al., 2012; Miller et al., 2010).

In primary B cells from tonsillar tissue, the expression of Jmjd3 increases as cells become differentiated, with the highest expression observed in memory B cells. Furthermore, Jmjd3

over-expression experiments in transfected primary B cells showed that the transcriptional targets of Jmjd3 are indeed genes differentially expressed in plasma cells and memory cells. This supports a role for Jmjd3 in B cell differentiation. Jmjd3 was even further up-regulated in B cells infected with the oncogenic Epstein-Barr virus (EBV) and in malignant Reed-Sternberg cells (derived from B lymphocytes) from Hodgkin's lymphoma tumors (a type of cancer developed in some cases secondary to EBV infection). Since Jmjd3 up-regulation is caused by the latent membrane protein 1 (LMP1), which is the major EBV transforming gene, and both proteins share transcriptional target genes, this suggests that LMP1- induced malignant transformation can be partially mediated by Jmjd3 (Anderton et al., 2011).

Jmjd3 has also been implicated in the pathogenesis of autoimmune diseases. In anti-neutrophil cytoplasmic autoantibody (ANCA) vasculitis, autoantibodies are mainly directed against the early neutrophil granule proteins, proteinase 3 (PR3) and myeloperoxidase (MPO), which are abnormally expressed in mature neutrophils. The up-regulation of PR3 and MPO correlated with a decrease in H3K27me3 at their promoters and with a concomitant elevation of JMJD3 in neutrophils from ANCA patients compared with healthy controls. Together these observations suggest that Jmjd3 reverses the normal silent state of PR3 and MPO in differentiated cells by erasing the repressive histone mark (Ciavatta et al., 2010). Contrarily, reduced Jmjd3 binding seems to contribute to the pathological mechanisms behind Systemic lupus erythematosus (SLE), an autoimmune disease characterised by anti nuclear auto-antibodies and T cell over-activation. In $CD4^+$ T cells from patients with SLE, the decreased expression of hematopoietic progenitor kinase 1 (HPK1), a negative regulator of TCR signalling, is associated with increased H3K27me3 levels and loss of Jmjd3 co-localisation at its promoter, ultimately leading to T cell hyper-reactivity and B cell overstimulation (Zhang et al., 2011).

In summary, Jmjd3 up-regulation allows differentiated cells to reprogram their chromatin in response to environmental factors, providing a mechanism linking inflammation to the control of gene expression and cell differentiation (De Santa et al., 2007).

1.2.3.1. Jmjd3 regulation of gene expression

In *Jmjd3* knockout LPS-activated primary macrophages, De Santa *et al.* described a reduction in the mRNA levels of *Ccl5*, *Il12b*, *Cxcl11* and *Ccl9* among other genes. This mRNA reduction was associated with a decrease in Pol II recruitment to the TSS and in the levels of nascent chromatin-associated RNA, reflecting a reduction in the number of elongating polymerases across the genes. These findings hint at a role for Jmjd3 in transcription initiation and elongation, independent of its demethylase activity (De Santa et al., 2009). A recent report using co-immunoprecipitation assays in 293 T and in macrophage-like differentiated HL-60 cells confirmed that Jmjd3 associates with elongation factors including: SPT6, SPT16, CDC73, the H3K36 methyltransferase SET2 and the H3K27me2 demethylase KIAA1718. In the macrophage-like differentiated HL-60 cells, Jmjd3 and KIAA1718 cooperate to demethylate H3K27me3 at the promoter of a group of shared target genes, thereby increasing their expression. More importantly, Jmjd3 and KIAA1718 were also found binding to the bodies of their targets genes and knockdown experiments corroborated that the two enzymes contribute to the recruitment of positive elongation factors to these genes. This in turn correlated with an increase of RNA pol II throughout the gene bodies (Chen et al., 2012). Together these data suggests that at least at some target genes, Jmjd3 might be part of a protein complex that activates transcription by promoting Pol II progression along the gene bodies and transcriptional elongation.

It has also been shown that in terminally differentiated cells Jmjd3 has a role in chromatin remodelling which is not related to its H3K27me3 demethylase activity. Jmjd3 makes the

promoter region of target genes such as *Ifng* more accessible for transcription by providing a bridge for the interaction of T-box transcription factors, such as T-bet in T_H1 cells and a Brg1-containing SWI/SNF chromatin remodelling complex (Miller et al., 2010).

Therefore, specific gene promoters and/or specific cell differentiation stages or cell types may require Jmjd3's H3K27me3 demethylase activity, the transcriptional elongation regulation function or the chromatin remodelling role to regulate gene expression.

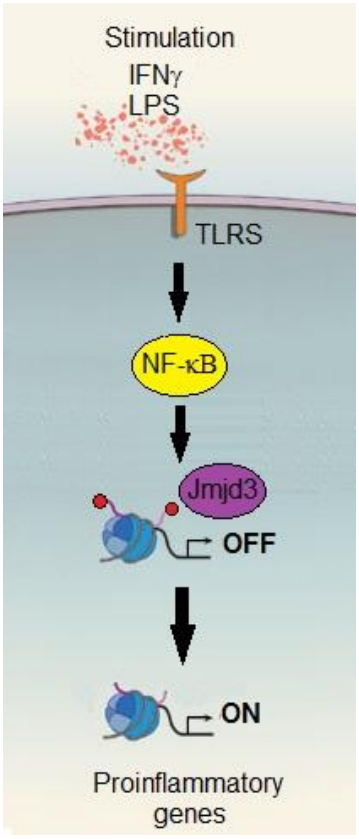


Figure 1.2. Jmjd3 regulation of gene expression. Following activation with pro-inflammatory stimuli (such as LPS and IFNγ), Jmjd3 is induced by the transcription factor NF-κB. Jmjd3 promotes the transcription of target genes (such as pro-inflammatory genes) either by removing H3K27me3 (transcriptional repressive histone mark), aiding chromatin remodelling or contributing to transcriptional elongation. Modified from (Foster et al., 2007).

1.2.4. Epigenetics in MS

Epigenetics is a young field and therefore the relation between epigenetic mechanisms and human diseases, maybe with the exception of cancer, is just starting to be determined.

Since epigenetic mechanisms can modulate the gene expression and cell phenotype in response to external stimuli (Jaenisch and Bird), the role of some susceptibility factors (especially environmental ones) in the pathogenesis of MS could perhaps be partially explained by such epigenetic mechanisms (Huynh and Casaccia, 2013; Koch et al., 2013).

The high discordance between identical twins (Sadovnick et al., 1993), the more common mother to daughter "transmission" of MS (Chao et al., 2010), the eminent importance of environmental exposure before adolescence (as reflected by the change in MS risk in migrants under 15 to the new geographical region incidence) (Dean and Elian, 1997), and the broad histopathological and clinical variation between patients (Lassmann et al., 2001) further support the involvement of epigenetic changes in MS.

1.2.4.1. MS and DNA methylation

The methylation of cytosines in the DNA interfere with the binding of transcription factors to the DNA and produce repression of transcription (Klose and Bird, 2006).

In a study carried out in three identical twin pairs discordant for MS not matched for sex and age, the DNA methylation pattern of blood isolated $CD4^+$ T cells showed no difference between the healthy and corresponding MS affected twin (Baranzini et al., 2010).

DNA methylation could contribute to increase the number of T_H17 cells and therefore inflammation in MS patients. A study in the methylation profile of $CD4^+$ T cells and its relation with lineage commitment showed that the promoter of *IL17A* is hypomethylated and therefore transcriptionally more active in T cells from MS patients, which could cause a skew of T cell differentiation towards a T_H17 phenotype (Janson et al., 2011).

Hypomethylation of the promoter of the enzyme peptidyl arginine deiminase type-2 (PAD2) that citrullinates MBP results in an overexpression of the enzyme and higher levels of citrullinated MBP in the NAWM of patients with progressive MS. Citrullinated MBP is less stable than regular MBP, which could contribute to myelin sheath instability and increased breakdown, releasing antigenic peptides and contributing to the development of an autoimmune response (Mastronardi et al., 2007; Moscarello et al., 1994).

Another study investigating the relation between DNA methylation and changes in disease activity in RRMS patients compared the methylation status of 56 gene promoters in cell-free plasma DNA from relapsing patients, patients in remission and healthy controls. 15 of 56 genes were differentially methylated between MS patients and controls, while in 5 of these genes the methylation level of the promoter allowed the differentiation between patients in a relapse and patients in remission with 70.8% sensitivity and 71.2% specificity (Liggett et al., 2010).

Further studies, with a bigger sample size, analysing the DNA methylation profile in different cell types and tissues from MS patients compared to healthy controls are needed to analyse the contribution of DNA methylation at specific genes in MS pathogenesis. Likewise, further repetitions and larger sample size are needed in order to establish the validity of DNA methylation of specific genes as a potential biomarker of disease activity (Huynh and Casaccia, 2013; Koch et al., 2013).

1.2.4.2. MS and histone modifications

The only histone modification that has been studied in relation to MS is histone acetylation. Histone acetylation promotes the expression of genes by neutralizing the negative charge of DNA and disrupting its binding to histones, making chromatin more open and available for the transcription machinery (Kouzarides, 2007). In particular, the link between histone

acetylation, oligodendrocyte precursor cells differentiation and re-myelination of affected axons has been established.

During normal development the myelination of axons requires the down-regulation of transcription factors that inhibit oligodendrocyte differentiation. Such down-regulation is achieved by the increased expression of histone deacetylases (HDACs) which catalyse the removal of histone 3 (H3) acetylation (a marker of transcriptional activation) from the promoter of differentiation inhibitors (Shen et al., 2005).

H3 deacetylation is also important for the myelin repair observed after demyelination induced by cuprizone in a mouse model. Adult oligodendrocyte progenitors' differentiation is accompanied by an increased activity of HDACs which repress the expression of differentiation inhibitors. In the same cuprizone model of demyelination, old mice showed a reduced ability to up-regulate HDACs in olygodendrocyte progenitors. This reduced expression of HDACs was accompanied by a reduction of their recruitment to the promoter of Sox2 (stem cell transcription factor) and transcriptional inhibitors, which caused their persistent expression and blocked the differentiation of oligodendrocyte precursors, impairing re-myelination. The HDAC inhibitor, valproic acid, blocked the differentiation of oligodendrocyte precursors and re-myelination when administered to young mice by the same mechanism as advanced age. Therefore, age dependent inefficient remyelination seems to be linked to a failed HDAC activity (Shen et al., 2008).

In a study with samples from progressive MS patients, oligodendrocytes in the NAWM of MS patients showed a higher level of H3 acetylation compared to controls, which was associated with high levels of transcriptional inhibitors of oligodendrocyte differentiation. This suggests histone acetylation has a pathophysiological role in MS progression by dampening remyelination and propitiating axonal damage. In contrast, oligodendrocytes in early MS lesions with ongoing demyelination and/or remyelination showed an important H3

deacetylation. Therefore, histone deacetylation of oligodendrocytes occurs at the early stages of the disease, correlates with remyelination, and its efficiency decreases with disease duration. Additionally, similar to what has been described in mice, histone acetylation is increased in the white matter of aged control subjects (Pedre et al., 2011).

In EAE studies, treatment with histone deacetylase inhibitors such as sodium phenylacetate and trichostatin A ameliorates disease and inflammatory infiltrates (Camelo et al., 2005; Dasgupta et al., 2003). However, as discussed before, deacetylation in oligodendrocyte progenitors is essential for the differentiation into mature cells and the repair of myelin, which could negate other putative positive effects of HDACs inhibitors in MS (where demyelination is more prominent than in murine models).

Although some epigenetically regulated mechanisms involved in the pathophysiology of MS that contribute to increased susceptibility have been elucidated, there is still a lot of research to be done in this respect. Until now, no successful treatment has been developed for secondary progressive MS and targeting microglial activation could be a good therapeutic option. Deciphering the epigenetic phenotype of microglia in MS and investigating whether epigenetic changes are involved in the maintenance of a gene expression program that contributes to the persistent activation of microglia in the late stages of the disease could lead to the development of new therapeutic interventions.

Since it seems that Jmjd3 allows terminally differentiated cells to reprogram their chromatin, and thus their gene expression profile in response to their micro-environment, it is a good candidate to study in relation to chronic microglial activation in MS and EAE.

1.3. Hypothesis

Increased expression of the histone demethylase, Jmjd3, in microglia leads to up-regulation of genes that contribute towards the initiation and maintenance of the pathological chronic activated state seen during remissions and the progressive stage of MS and EAE.

1.4. Thesis aims

- Examine the gene and protein expression of Jmjd3 by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical analysis in activated microglia stimulated with bacterial products and pro-inflammatory cytokines using a microglial cell line and primary murine microglia.

- Determine the target genes of Jmjd3 implicated in the induction and maintenance of persistent activation in primary murine microglia using chromatin immunoprecipitation (ChIP) along with qRT-PCR.

- Investigate if the binding of Jmjd3 to target genes in activated primary murine microglia has an influence on the levels of the H3K27me3 mark, which may result in an increase in the transcription of such genes.

- Examine the expression of Jmjd3 at the mRNA and protein levels in surveying ("resting") and activated microglia/infiltrating macrophages isolated from naïve versus EAE mice brain and spinal cord, using qRT-PCR and flow cytometry.

- Investigate if there is a difference in the expression of Jmjd3 in activated microglia/CNS infiltrating macrophages between relapses and remissions in a CR-EAE Biozzi mice model, using qRT-PCR and flow cytometry.

- Determine the functional consequences of blocking/reducing the expression of *Jmjd3* with ShRNA, or through the use of *Jmjd3* knockout mice primary microglia, by examining the expression of target genes in activated primary microglia *in vitro*.

2. Materials and methods

2.1. Cell culture

2.1.1. Cell lines

HEK 293T, EOC 20 and LADMAC cells were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured at 37°C in a 5% CO^2 humidified atmosphere.

HEK 293T cells and LADMAC cells were maintained in Dulbecco's Modified Essential Medium (DMEM; Sigma-Aldrich), supplemented with filter-sterilised 10% (v/v) foetal calf serum (FCS; Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich).

EOC 20 cells were cultured in DMEM supplemented with 10% FCS (v/v), 100 U/ml penicillin-streptomycin and 20% LADMAC conditioned media.

HEK 293T and EOC 20 adherent cells were passaged when they reached 70-80% confluency. Non-adherent LADMAC cells were passaged when they reached a density of 1-2 x 10^6 cells/ml. HEK 293T cells were re-suspended into the media by trypsinisation (Lonza). EOC 20 cells were dislodged using a cell scraper. The cell suspension was centrifuged at 1000 x *g* for 5 min and re-suspended in culture media for counting.

2.1.2. Cell storage

Cell lines were stored at a density of 2-3 x 10^6 cells/ml in FCS containing 10% (v/v) dimethyl sulphoxide (DMSO; Sigma-Aldrich) at -80° C or -196° C (liquid nitrogen). Cells were thawed in a water bath at 37°C and diluted drop-wise into 10 ml pre-warmed culture media. The cells

were then centrifuged at 900 x *g* for 5 min and then re-suspended in culture media for counting after which cells were seeded at an appropriate density in culture flasks.

2.1.3. Cell quantification

Cell concentrations were determined manually using a haemocytometer. Cell viability was determined by Trypan Blue (Sigma-Aldrich) staining.

2.1.4. LADMAC conditioned media production

2 x 10^5/ml LADMAC cells were cultured in 150 cm^2 culture flasks with 150 ml of DMEM, supplemented with 10% FCS and 100 U/ml penicillin-streptomycin. The flasks were incubated for 1 week, during which time the cultures become confluent and the cells secrete CSF-1 into the media. Subsequently, the media was collected, centrifuged at 500 x *g* for 30 min, sterile filtered with 0.22 μm filters and stored in 50 ml aliquots at -20°C for further use.

2.1.5. Primary murine microglial cell culture

To prepare mixed glial cultures 0-2 day old pup brains were dissected and placed into sterile phosphate buffer solution (PBS) on ice. Meninges were quickly removed using light microscopy. Brains were minced into small pieces using a scalpel and collected into 15 ml conical tubes (2-5 brains per tube) with 10 ml of sterile filtered Hanks' balanced salt solution (HBSS; Sigma-Aldrich) plus 50 mM EDTA and 30-40u/ml of papain (Worthington) and incubated at 37°C for 20 min on a rotating platform. Digested brains were centrifuged at 100 x *g* for 2 min and supernatants were discarded. Cell pellets were triturated in 1 ml of Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12; Sigma-Aldrich) with 10% FCS and 1.5 mg/ml of bovine serum albumin (BSA; Sigma-Aldrich), pipetting up and down gently with a P1000 pipette to obtain an emulsion. Cell suspensions were diluted in DMEM-F12 supplemented

with 10% FCS, 100 U/ml penicillin-streptomycin and 0.5 µg/ml of Fungizone (Gibco) and plated onto 6-well plates or 25 cm^2 culture flasks at a density of ~90,000 cells/cm^2 and cultured at 37°C in a 5% CO2 humidified atmosphere. Cultures were washed the next day with HBSS to remove cellular debris and fed every 3 days with a 50% replacement media. The isolated media, corresponding to astrocyte-conditioned media (ACM), was stored at 4°C for further use on the microglia culture post-trypsin stripping. After 15–30 days (once a monolayer was formed), the DMEM-F12 was removed, cultures were washed with warmed HBSS and incubated at 37°C in a 5% CO_2 incubator with 2 ml per well or 2.5 ml per 25 cm^2 culture flasks of 0.25% trypsin solution (HBSS, 0.5 ml 10x trypsin/EDTA solution, 50 mM EDTA, 100 mM $CaCl_2$, diluted at 1:3 with DMEM-F12). Cultures were monitored every 15 min until the monolayer was detached, after which 2 ml of DMEM-F12 supplemented with 10% FCS per well were added for trypsin inactivation. The media containing the layer of detached cells was aspirated and discarded. The remaining attached cells on the 6-well plates or 25 cm^2 culture flasks, corresponding to the microglia, were washed with warmed HBSS and 2 or 4 ml of fresh DMEM-F12 supplemented with 10% FCS, 100 U/ml penicillin-streptomycin and 0.5 µg/ml of Fungizone mixed with 50% ACM were applied. The new microglia primary culture was left to equilibrate for 24-48 hours before subjecting it to experimental conditions. (Saura et al., 2003). The number of microglial cells obtained after trypsinisation of mixed glial cultures was ~1.5-2 x 105 cells per well on the 6-well plates and ~4-5 x 105 cells per 25 cm2 culture flask. To determine the purity of the microglial cultures, the cells were stained with FITC conjugated anti-mouse CD45.2 (BD Pharmigen) and APC conjugated anti-mouse CD11b (eBioscience) antibodies and then analysed by flow cytometry. All cultures had a purity higher than 95%, as determined by the percentage of CD11b+ CD45+ cells.

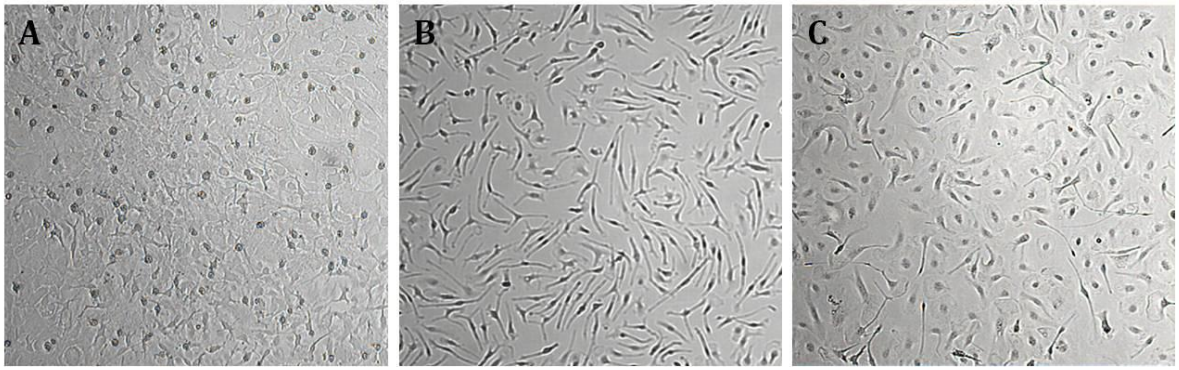


Figure 2.1. Light micrographs of primary murine mixed glial and microglial cultures. (A) Confluent murine mixed glial culture prior to mild trypsinisation. (B) Primary microglial culture 48 hours after trypsinisation. (C) Primary microglial culture treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2 hours. After activation the primary microglia acquire a de-ramified, rounder morphology. 40x magnification.

2.1.6. EOC 20 cells and primary murine microglia stimulations

EOC 20 cells and/or primary murine microglial cells were left untreated or stimulated with lipopolysaccharides from E.Coli serotype 055:B5 (LPS; Sigma-Aldrich), recombinant mouse IFN-γ (BD Pharmingen), FSL-1 (InvivoGen), Pam3CSK4 (InvivoGen), Imiquinoid (InvivoGen), ODN1826 CpG oligonucleotide (InvivoGen) and Poly (I:C) (InvivoGen). After incubation the media was aspirated, cells washed with 37°C warmed PBS, scraped and pelleted by centrifugation at 300 x *g* for the EOC 20 cells and at 100 x *g* for the primary microglia for 5 min for RNA isolation.

2.1.7. Transient transfection

2 x 10^5 HEK 293T cells were seeded per well in a 6-well plate or 2 x 10^6 cells per 75 cm^2 culture flask one day before transfection. For each well, 100 µl of Opti-MEM serum-free media (Invitrogen) was mixed with 3 µl GeneJuice (Novagen) an incubated at room temperature for 5 min. For the 75 cm^2 culture flasks, 2 ml of serum-free media were mixed with 20 µl of GeneJuice. For complex formation 3 µg of plasmid DNA (pCMV-*Jmjd3*-HA) per well or 7 µg per

75 cm^2 culture flask were added and the GeneJuice/DNA mixture was incubated at room temperature for 15 min. The complexes formed were then added drop-wise to the cells in complete growth media and mixed by gently rocking the plate/flask. After 48 hours incubation at 37°C in 5% CO2 cells were analyzed for expression or used in downstream applications.

2.2. Flow cytometry

2.2.1. Surface staining

Cells were stained in V-bottomed, 96-well plates (BD Falcon) at 4°C in flow cytometry buffer (PBS with 2% FCS) with either fluorescent conjugated primary antibodies, or with unconjugated primary antibodies followed by staining with fluorescent conjugated secondary antibodies for 20 min. Cells were then washed twice with 200 µL of flow cytometry buffer and pelleted by centrifugation at 400 x g at 4°C for 2 min. Pellets were then re-suspended in 500 µL flow cytometry buffer and analysed with a FACS (fluorescence activated cell sorting) Calibur (BD Biosciences). Data was analysed using Summit 4.3 software.

2.2.2. Intracellular staining for flow cytometry

For the Jmjd3 intracellular staining, cells were permeabilised with 250 µl permealisation buffer (one part of fixation/permeabilisation concentrate plus three parts of fixation/permeabilisation diluents (eBioscience)). Cells were incubated for 18 hours at 4°C and then washed twice with 1x permeabilisation buffer (eBioscience) (diluted with H_2O from the 10x stock). Cells were stained with a rabbit anti-mouse Jmjd3 polyclonal antibody (kindly provided by Dr. Gioacchino Natoli, IFOM-IEO Campus, Milan, Italy) diluted 1:100 in 1x permeabilisation buffer and incubated for 30 min at 4°C. Cells were washed twice with 1x

permeabilisation buffer and 1 µg/million cells of purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block, BD Pharmingen) diluted in 50 µl of 1x permeabilisation buffer were added per well and incubated for 5 min at 4°C. Without washing, the cells were then stained with 10 µg/mL of the secondary fluorescent goat anti-rabbit antibody Alexa Fluor 488 (Molecular probes) in 50 µl of 1x permeabilisation buffer and incubated for 30 min at 4°C. After washing twice with 1x permeabilisation buffer following the staining, these cells were finally re-suspended in 500 µl of FACS buffer and analysed with a FACS Calibur (BD Biosciences). Data was analysed using Summit 4.3 software.

HEK 293T cells transiently transfected with the pCMV-HA-*Jmjd3* plasmid were also stained for the HA tag. For this anti-HA staining, cells were incubated with the monoclonal mouse anti-HA.11 primary antibody (clone 16B12, Covance) at a final concentration of 10 µg/ml for 45 min, at room temperature. Cells were then washed twice with flow cytometry buffer and incubated with APC-conjugated Goat-Anti-Mouse IgG (BD Bioscience) at a concentration of 0.4 µg/ml for 30 min at 4°C in the dark.

2.3. Lentivirus cell transduction

2.3.1. Lentivirus preparation and concentration

24 h before transfection, a confluent T75 culture flask of HEK 293T cells was split in a 1 to 5 ratio. The following day, when cells achieved a confluency of ~ 50%, 1.6 ml of Opti-MEM media (Gibco, Invitrogen) were mixed with 32 µl of TransIT®-293 Transfection Reagent (Mirus, Cambridge BioScience) in a universal tube and incubated at room temperature for 5 min. In a separate tube, 4 µg of psPAX2 (2nd generation packaging vector), 4 µg of pMD2.G (VSV-G envelope plasmid) and 6 µg of the desired pGIPz shRNAmir transfer vector were mixed and then added drop-wise to the OPTI-MEM-TransIT®-293 mixture. To allow

complexes to form, the solution was incubated at room temperature for 15 min, after which 12 ml of pre-warmed complete cell culture media was added. The media in the 293 T cell flask was replaced with this transfection media and the cells were incubated at 37°C in a 5% CO^2 humidified atmosphere for 48 h. The viral supernatants were harvested at 48 h (when another 12 ml of pre-warmed complete DMEM media were carefully added to the cells) and 72 h post-transfection and stored at 4°C. The pooled viral supernatants were then centrifuged at 1000 x *g* for 5 min, sterile-filtered through a 0.2 µm filter to remove cell debris into sterile ultra-centrifuge tubes (Beckman-Coulter) and weighted to balance them. The viral particles were concentrated by ultracentrifugation at 22 000 *x g* at 4°C for 2 h 30 min using an Avanti JA 30.50 rotor. The supernatant was discarded and the lentiviral pellet re-suspended in 150-200 µl of HBSS using sterile filter tips, aliquoted and stored at -80°C. To determine the viral titer, HEK 293T cells were seeded at a concentration of 1 x 10^5 cells in 1 ml per well of a 12 well plate and five 10-fold serial dilutions of the concentrated virus were added to different wells. Cells were then incubated at 37°C in a 5% CO^2 humidified atmosphere for 72 h. To assess the transduction efficiency, the percentage of GFP positive cells was quantified by flow cytometry. The viral titer in transduction units (TU/µl) was calculated according to the following formula: TU/µl = (P * N / 100 * V) * 1/DF, where P = % GFP^+ cells, N = number of cells at time of transduction (10^5), V = volume of dilution added to each well (20 µl) and DF = dilution factor [1 (undiluted), 10^{-1} (diluted 1/10), 10^{-2} (diluted 1/100), and so on].

2.3.2. Primary microglia transduction

Primary murine microglial cultures were transduced with the packaged lentiviral particles at different multiplicities of infection (10, 100, 250, 500 and 1000) and incubation times (24, 48 and 72 h), with or without adding polybrene to a final concentration of 8 µg/ml. After incubation with the lentiviral particles the media was replaced and the efficiency of

transduction was assessed by quantifying the percentage of GFP positive cells through flow cytometry.

2.3.3. Magnetic transduction (magnetofection)

For magnetic transduction, 30 µl of ExpressMag Beads (Sigma-Aldrich) were mixed thoroughly by pipetting with the lentiviral particles to a final volume of 200 µl (or 2-5 x 10^5 primary microglia cells/well of a 6-well plate). The ExpressMag and virus cocktail was incubated at room temperature for 15 min to allow conjugation, after which it was added to the cells plus 1.8 ml of complete media per well. The 6-well plate was placed on top of the magnet for 30 min at 37°C in a 5% CO^2 humidified atmosphere. After this incubation the cell culture media was replaced and the efficiency of transduction was determined as previously stated.

2.4. Molecular Biology

2.4.1. RNA isolation

Total RNA was isolated using the RNeasy Micro Kit (Qiagen). Briefly, samples were homogenised in the RLT buffer by passing them 5 times through a 20 G needle fitted with an RNase-free syringe. One volume of 70% ethanol was added, the sample applied to an RNeasy MinElute spin column and centrifuged at 10,000 x *g* for 1 minute. On-column DNA digestion was performed to remove contaminating genomic DNA. The column was washed with buffer RW1, buffer RPE and 80% ethanol. RNA was eluted in 14 µl of RNase-free water (Sigma-Aldrich). RNA quantity and quality was determined by measuring the ratio of absorption obtained at 260 nm and 280 nm using a NanoDrop (Thermo-Scientific, USA).

2.4.2. cDNA synthesis

RNA (1 μg from the EOC 20 cells, 100 ng from primary murine microglia and 50 ng from the Biozzi mice EAE samples) was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). To control for any genomic DNA contamination in the reaction, samples in which the reverse transcriptase enzyme was replaced with water, were also included. The reaction composition was as follows:

Reagent	***Final Concentration/Volume***
RT Buffer (2X)	1X
RT Enzyme (20X)	1X
RNA sample	up to 2 μg
RNAse-free H_2O	to 20 μL

Reverse transcription was performed on a DNA Engine Tetrad (MJ Research) using the following program:

Step	***Temperature (°C)***	***Time (min)***
1	37	60
2	95	5
3	4	∞

2.4.3. Quantitative real-time polymerase chain reactions (qRT-PCR)

For the JmjC-domain containing proteins screening and the microglial activation marker genes qRT-PCR reactions were performed using Power SYBR green master mix (Applied Biosystems) and custom-made primers. The sequences of the primers used for the 25 JmjC-domain proteins analysed were the same as published by De Santa et al. (De Santa et al., 2007)

(Table 2.1). The primers for the microglial immune response and inflammation related genes were custom designed using Primer3 Plus (Table 2.2).

Expression levels of *Jmjd3*, and *Utx* were analysed using TaqMan Gene Expression Assays (Applied Biosystems). For the Taqman gene expression assays, qRT-PCR reactions were carried out in a final volume of 20 µl, containing 2 µl of cDNA, 1 µl of 20X Taqman gene expression assay and 10 µl of Taqman universal PCR master mix (Applied Biosystems). For the Power SYBR green master mix and custom-made primers, qRT-PCR reactions were carried out in a final volume of 25 µl, containing 2 µl of cDNA, 300-400 nM of 5µM forward and reverse primers and 12.5 µl of 2X Power SYBR green master mix. Reactions were performed in triplicate using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Data was analysed using the SDS v. 2.4 software (Applied Biosystems). Expression levels (given as fold induction), normalised to the house-keeping gene, Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), and relative to a calibrator (non stimulated sample), were calculated using the formula, $2^{-\Delta\Delta CT}$. The ΔΔCT value is obtained by subtracting the cycle threshold value (Ct) for *Hprt* from the Ct value for each target (ΔCT), and then subtracting the normalised Ct value of the calibrator from the normalised Ct value of each target (ΔΔCT). The efficiency value used was 2 since both target and control transcripts PCR amplification efficiency was close to 100% (calculated from the slope of a standard curve made with a serial dilution of the targets in the case of the custom-made primers).

Gene	Sense primer	Antisense primer
Fbxl10	GAGGTGTGGATGGCAGTCTT	CCAACTGAGGTCAAGGGAGA
Fbxl11	CAGGTTGGATTCATGCTGTG	GGATCGGTTGGTTATGCAGT
Hr	TGTCAGGCATCCAGAAGACA	GTTCGGTGTAGCAGGAGGAC
Hspbap1	GGTGGCATTACGTGGAGTCT	CAGCTGTTGACCTCGTGAGA
Hif1an	TCATTGGCATGGAAGGAAAT	GAAATTGGGGAACCTCTCGT
Jmjd1a	CACATTTAGGTTCCCAGTCACA	GCCACGATGTTAACACAGGA
Jmjd1b	TTCTGCTGGAAGGCTCACTT	GATGCATCCCATTAGCATCC
Jmjd1c	AGAAGAGGAAAGGCGAGGTC	TTGGGACCTATCTCACAGCA
Jmjd2a	GACCACACTCTGCCCACAC	TCCTGGGGTATTTCCAGACA
Jmjd2b	GGCTTTAACTGCGCTGAGTC	GTGTGGTCCAGCACTGTGAG
Jmjd2c	CACGGAGGACATGGATCTCT	CGAAGGGAATGCCATACTTC
Jmjd2d	GTCTTGGTCGTCGTCCTTGT	AATCCCCCTTCAGAAGCTGT
Jmjd3	CACTTCCAACTCCATCTG	AATCACAAGACTTCTCTATCC
Jmjd4	CTCAAGGACTGGCATCTGTG	CTGAAGGAGCGGAAGATGTC
Jmjd5	TGTCATGTTAGAGCGGATGG	TGTACCTTGAGCCCACTTCC
Jarid1a	CCTCCATTTGCCTGTGAAGT	CCTTTGCTGGCAACAATCTT
Jarid1b	AGAGGCTGAATGAGCTGGAG	TGGCAATTTTGGTCCATTTT
Jarid1c	GGTGAGCCAAAAACCTGGTA	CAAATTCTCCTGCACACTGG
Jarid1d	CCCCAAGGGAGTATGGAGAT	GCCTCCAGAATTCCTTTTCC
Jarid2	GGTCTGCTCAGGACTTACGG	TTGGGTTTGGTTTCCTTGAC
Mina	GCAAAGGAAGATGTGGCATT	CCTCGAAGAAAGGAGGGAGT
Phf2	GCCAAAGAGATCAGGCTCAG	TGGGCATCTTCACAGTCTTG
Phf8	CGCCCAACAAATGCTAATCT	AGAAGTTCCCTCCGAATGCT
Ptdsr	AAGGAGGGAACCAACAGGAT	CTGGCAAAGTTCTGGGTGAT
Utx	ATCCCAGCTCAGCAGAAGTT	GGAGGAAAGAAAGCATCACG

Table 2.1. Primers for the JmjC-domain containing proteins qRT-PCR screening.

Gene	Sense Primer	Antisense Primer
Ccl3	AGCCAGGTGTCATTTTCCTG	TCTCAGGCATTCAGTTCCAG
Ccl5	ACCATATGGCTCGGACACCACT	ACCCACTTCTTCTCTGGGTTGG
Ccl9	TGGGTCTGCCCACTAAGAAG	TAGGACAGGCAGCAATCTGA
Cxcl11	GCTGCTGAGATGAACAGGAA	ACTTTGTCGCAGCCGTTACT
Il6	CCATAGCTACCTGGAGTACATG	TGGAAATTGGGGTAGGAAGGAC
Il12b	TCTGAGCCACTCACATCTGC	TCAGGGGAACTGCTACTGCT
Nos2	CCATCATGAACCCCAAGAGT	CATCCAGAGTGAGCTGGTAGG
TNFα	ACGTCGTAGCAAACCACCAA	GCAGCCTTGTCCCTTGAAGA

Table 2.2. Primers for the microglial immune response and inflammation related genes.

2.4.4. Chromatin immunoprecipitation- quantitative real-time PCR (ChIP-qPCR)

Firstly, 5 x 10^7 EOC 20 cells or 1 x 10^7 primary murine microglial cells were stimulated for 2 hr with 100 ng/ml of LPS plus 100 U/ml of IFNγ. After stimulation, the cells were washed with PBS, dislodged using a cell scraper and centrifuged at 100 x *g* for 5 min at 4°C. The cell pellet was re-suspended and fixed by adding 10 ml, 1% formaldehyde (prepared from a 36.5% formaldehyde stock diluted with PBS; VWR) and cross-linked for 10 min at room temperature. The reaction was stopped by adding 1.25 ml fresh 1M Glycine (final concentration 0.125 M;VWR) and incubating for 5 min at room temperature. Cells were washed twice with 10 ml of ice cold 1x PBS plus 400µl complete protease inhibitors (Roche) and centrifuged at 400 x *g* for 5 min at 4°C (supernatant was discarded between washes). After centrifugation, cells were lysed in 100 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8) per 1 x 10^7 cells, supplemented with 400 µl of complete protease inhibitors (Roche) and incubated for 10 min on ice. The sonication conditions were optimised beforehand using EOC 20 cells and primary murine resident peritoneal macrophages to yield chromatin fragments between 200 and 750 bp (Figure 2.2). Chromatin was sheared by sonication for 10 min at 4°C in a Covaris S-Series sonicator in 100 µl micro-tubes (Covaris). The operating conditions were: duty cycle 2%, intensity 3, cycles per burst 200, frequent sweeping mode and continuous degassing. The sonicated samples were then centrifuged at 20 000 x *g* for 10 min at 4°C to pellet debris. The supernatant was transferred to a new tube and diluted 10 times with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8 and 167 mM NaCl) supplemented with protease inhibitors. To pre-clear and reduce background, 2µl of a 1:1 mixture of Dynabeads protein A and Dynabeads protein G (Invitrogen) rinsed in dilution buffer were added to the samples and then rotated for 30 min at 4°C, followed by centrifugation at 100 x *g* for 1 min. The supernatants were removed and transferred to a new tube. Before proceeding, a 50µl aliquot from the supernatant was taken out as input and

stored at -20°C. A further 50µl (5% of the sample) aliquot was set aside for sonication control and de-crosslinked for a minimum of 3 hr with 150 µl of elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA and 1%SDS). The DNA was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and resolved by electrophoresis in a 1% agarose gel. Immunoprecipitations were carried out at 4°C overnight with 5 µg of anti mouse IgG rabbit polyclonal antibody (Abcam), 5 µg of anti mouse C-terminus Jmjd3 rabbit polyclonal antibody (kindly provided by Dr. Gioacchino Natoli, IFOM-IEO Campus, Milan, Italy), 5 µg of anti mouse H3K4me3 rabbit polyclonal antibody (Millipore Upstate) and 5 µg of anti mouse H3K27me3 rabbit polyclonal antibody (Millipore Upstate) per 1 ml of cleared chromatin. Immune complexes were collected by incubation for 3 to 5 hr with rotation at 4°C with 10µl a 1:1 mixture of Dynabeads protein A and Dynabeads protein G. Dynabeads were washed 3x with 1 ml of RIPA washing buffer (50 mM Hepes-KOH pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40 and 0.7% Na-deoxycholate) and 1x with 1 ml of TE (10 mM Tris-Cl pH 7.5, 1mM EDTA) plus 50 mM NaCl. For all the washes the Dynabeds were re-suspended in wash buffer and the tubes were then placed on a magnetic stand. The wash buffer was removed after 30 s to allow the beads to collect on the side of the tube. After the last wash the samples were centrifuged for 3 min at 960 x *g*, and the buffer TE was carefully removed with a pipette. Dynabeads were then eluted with 210 µl of fresh elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA and 1% SDS) and incubated for 30 min at 65°C with shaking. Samples were centrifuged for 1 min at 16,000 x *g* at room temperature and 200 µl of supernatant was removed and transferred to a new tube. The input sample was diluted to 200 µl by adding 150 µl of elution buffer and was incubated at 65°C overnight with the immunoprecipitated samples to reverse crosslinks. The next day, the DNA was purified with PCR purification columns (Qiagen), according to the manufacturer´s instructions and eluted in 50 µl of H_2O. 2µl of immunoprecipitated DNA were used per qPCR reaction with primers specific for the

promoter region of selected immune response and inflammation related genes (Table 2.3). For the ChIPs with primary murine microglia, the chromatin enrichment was calculated as percentage of input following the equation $2^{-\Delta Ct}$(5). To calculate the ΔCt (normalised to the input samples), input Ct values were subtracted from the ChIP samples Ct values and then multiplied by 5 (fraction of the input chromatin saved). In the case of the EOC 20 cells ChIPs, when a much higher number of cells were available, a nonspecific IgG antibody control sample was included.

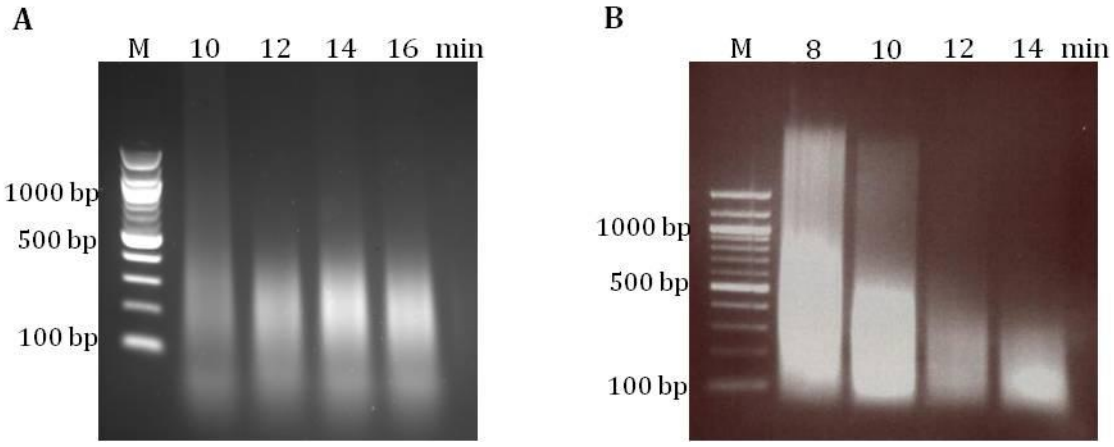


Figure 2.2. Sonication optimisation. A total of $1x10^7$ crosslinked and lysed EOC 20 cells (A) or primary murine resident peritoneal macrophages (B) were sonicated with a Covaris S-Series sonicator for various amounts of time. After sonication, the crosslinks were reversed, DNA purified and run on a 1% agarose gel. Lanes with sonicated material are labelled with the total time of sonication in minutes. Size of molecular marker (M) bands are indicated. Ten minutes provided a good degree of sonication.

Gene	*Sense primer*	*Antisense primer*
Ccl3	CACTCCAAACCCATTCCTTG	AGCTGGAGATGCTTTTCCTC
Ccl5	ACGGCTACCCTGTCATCACT	CGGCACCAGTTCACAATAAG
Cxcl11	GAAGCTACGGGCACCAAATA	TCCTCAGGTTCAGCCTTCCT
Il6	AGGAGTGTGAGGCAGAGAGC	GTCTCCTCTCCGGACTTGTG
Il12b	GGGAGTGGGGTAAGTGGATT	CTGGCCTTAAACTCCTGTGC
Nos2	GCTTGGGTCTTGTTCACTCC	AACTGGCAGACCCTTCTGTG
Gbp6	CCAGGCCATAAACTCCACAT	CTGGGCTGATATTCCCGTAG
Arhgef3_TSS1	CGCGTCTTTTAAACTGCACA	ACCAGGATAAGACGCTGCTG

Table 2.3. Primers used for ChIP-qPCR analysis.

2.5. Immunocytochemistry

Primary murine microglial cultures were prepared as described in section 2.1.5. Cell suspensions were plated at 1 x 10^5 cells/well onto sterile poly D lysine coated Ø 13 mm glass cover slips (VWR) in 12-well plates. Microglial cells were left untreated or stimulated *in vitro* with 100 ng/ml of lipopolysaccharides from *E.coli* (LPS; Sigma-Aldrich) plus 100 U/ml of interferon-γ (Recombinant mouse IFNγ; Becton Dickenson) for either 2 or 4 hr. Cells were then washed twice with PBS and fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer for 10 min at 4°C. After washing two more times with PBS, cells were permeabilised by adding 0.1 % Triton X-100 (Sigma-Aldrich), 2 % BSA in PBS for 10 min and then blocked for non-specific binding in blocking buffer [2% BSA, 5% normal goat serum (NGS; Dako), 0.1 % Triton X-100 in PBS] for 45 min at room temperature. Cells were stained overnight at 4°C with a rat anti mouse CD11b antibody (MCA711G; AbD Serotec) and with either an anti mouse C-terminus Jmjd3 rabbit polyclonal antibody (kindly provided by Dr. Gioacchino Natoli, IFOM-IEO Campus, Milan, Italy), an anti mouse H3K4me3 rabbit polyclonal antibody (Millipore Upstate) or an anti mouse H3K27me3 rabbit polyclonal antibody (Millipore Upstate), all diluted in 100µl of blocking buffer per cover slip**.** The next day cells were washed 4 times in PBS and incubated for 2 hr in the dark at room temperature with the secondary antibodies

goat anti-rat IgG-Alexa Fluor 488 and goat anti-rabbit IgG-Alexa Fluor 568 (Molecular Probes) diluted 1:1000 in 100µl of blocking buffer per cover slip. No primary antibody control cover slips were set up, that were incubated with the secondary antibodies only, to ensure that there was no non-specific binding of the secondary antibodies. After washing 4 times with PBS cells were counterstained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Molecular Probes - Invitrogen) diluted 1:1000 in 300 µl of blocking buffer per cover slip and incubated for 10 min at room temperature in the dark. Cells were washed 4 more times with PBS and then the cover-slips were carefully mounted to glass microscope slides using Dako Fluorescent Mounting Medium (Dako Cytomation). The slides were dried in the dark for at least 2 hr at 4°C before cells were visualised and pictures were taken with a LSM510 confocal microscope (Zeiss Laboratories). The images were minimally processed using Zen 2011 and Image J softwares and quantified using MetaMorph® software (Molecular Devices). An intensity threshold was set as 3 times the background level value, determined by no primary antibody control images. Signal above the threshold was considered positive, and % of average positive marker (e.g. Jmjd3) intensity per area outlined (e.g. $DAPI^{+ve}$ nuclei) was used as quantitative measurement.

2.6. Mice

XB814 mice harbouring a gene trap cassette inserted in the intron 1 of the *Jmjd3* gene in a C57BL/6 background were generated by Professor Gioacchino Natoli**,** European Institute of Oncology (IEO), IFOM-IEO Campus, Milan, Italy. Biozzi ABH female mice used for induction of of chronic-relapsing (CR) EAE were obtained from Harlan Laboratories, Netherlands. C57BL/6 mice for induction of acute EAE were obtained from the biomedical services (BMS) in Oxford.

All mice were maintained and bred at the BMS in Oxford, in accordance with Home Office regulations. All experiments were performed under Lars Fugger's Home Office project licence.

2.6.1. Genotyping

To discriminate heterozygous mice for the *Jmjd3* knockout allele ($XB814^{+/-}$) from homozygous mice for the WT allele ($XB814^{+/+}$), ear notch tissue samples were lysed overnight at 40°C in lysis buffer (50 mM Tris pH 8, 100 mM Na_2EDTA pH 8, 100 mM NaCl, 1% SDS). The lysate was centrifuged at 1500 x g for 30 min at 4°C and the DNA was then precipitated in 500 µl of cold isopropanol, followed by washes with 500 µl of 70% and 95% ethanol and re-suspended in 200 µl of water. A triplex PCR-based strategy designed by Burgold *et al.* was used to identify $XB814^{+/-}$ and $XB814^{+/+}$ mice (Figure 2.3) (Burgold et al., 2012). The PCR reaction composition was as follows:

Reagent	***Final concentration/Volume***
DNA template	~50 ng
5x Phusion HF	1X
dNTPs (10 mM)	200 µM
Forw1 (25 µM)	500 nM
Rev1 (25 µM)	500 nM
Rev2 (25 µM)	500 nM
Phusion® High-Fidelity DNA Polymerase (2 U/µl)	0.1 µl
PCR Grade H_2O	To 20 µl

PCR reactions were performed on a DNA Engine Tetrad (MJ Research) using the following program and primers:

Temperature	*Time*	*Number of cycles*
98 °C	30 sec	1
98 °C	10 sec	
69 °C	15 sec	35
72 °C	10 sec	
72 °C	5 min	1
4 °C	∞	

Primer name	*Sequence (5'- 3')*
Forw1	AGG ATA CAG GAG CCA CGC G
Rev1	TGA CTC TCC ACT CGA TCA CCC
Rev2	TCC GGA GCG GAT CTC AAA C

The PCR products were run on a 2% agarose (Lonza) gel and visualised using ultra-violet light. XB814$^{+/-}$ were bred to obtain embryos for primary microglial cultures or to maintain the colony.

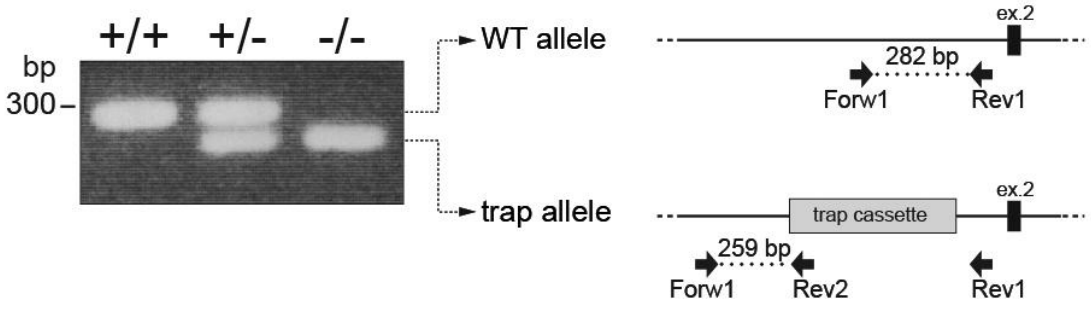


Figure 2.3. Triplex PCR-based genotyping strategy of XB814 mice. The scheme on the right shows the position of the three primers, with the Forw1/Rev1 product amplified from the wild type (WT) allele (282 bp), and the Forw1/Rev2 product amplified from the trapped (knockout) allele (259 bp). The agarose gel on the left shows the bands of the amplified products obtained from each genotype. (+/+)= WT homozygous. (+/-)=heterozygous. (-/-)= *Jmjd3* knockout homozygous. Figure obtained from (Burgold et al., 2012).

2.6.2. XB814 embryo genotyping

XB814$^{-/-}$ embryos (homozygous for the *Jmjd3* knockout allele) die at birth due to respiratory failure. Therefore to prepare primary microglial cultures from XB814$^{-/-}$ embryos, 18.5 day pregnant XB814$^{+/-}$ mice were sacrificed by exposure to carbon dioxide gas in a rising concentration and the embryos were quickly surgically removed from the uterine horns and placed in ice cold PBS. To identify XB814$^{-/-}$ embryos, tail biopsies were taken. DNA from the tail biopsies was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega), following the manufacturer's instructions and PCRs were carried out as described in section 2.3.1.

2.6.3. Induction and assessment of EAE in mice

C57BL/6 female mice aged between 7 to 9 weeks were used for induction of acute monophasic EAE. All EAE inductions were performed by the postdoctoral scientist Dr. Kate Attfield. 200 µg of MOG_{35-55} peptide (Sigma-Aldrich) dissolved in 200 µl of PBS per mouse was emulsified with 200 µl of complete Freud's adjuvant [incomplete Freund's adjuvant (Sigma) with *M. tuberculosis* H37RA (BD Biosciences)] containing 800 µg of *M. tuberculosis* per mouse. A total volume of 400 µl of the emulsion was injected sub-cutaneously (divided into 200 µl per flank) into each mouse. In addition, 200 ng of pertussis toxin (Sigma) in 200 µl of sterile PBS was administered intravenously on the day of immunization and 48 h later.

The mice were monitored daily following immunisation for weight loss and scored for neurological deficits using the following grading system for clinical assessment of EAE:

Score	Clinical signs
0	Normal mouse, no overt signs of disease
1	Weak/flaccid tail
2a	Limp tail
2b	Hind limb weakness, abnormal gait and/or impaired righting reflex
3	Partial hindlimb paralysis
4	Complete hindlimb paralysis

When mice reached a score of 2 or above, monitoring was increased to twice daily (early morning and late evening observations). Mice with weight loss greater than 20% of starting weight or that reached a score of 4 for more than 4 days were humanely sacrificed.

2.6.4. Induction and assessment of CR-EAE

To induce CR-EAE, Biozzi ABH female mice aged 7-9 weeks were injected subcutaneously in the flank with 200 µl of an emulsion composed of 300 µg MOG_{35-55} peptide mixed with incomplete Freund's adjuvant supplemented with 5 mg/ml *Mycobacterium tuberculosis* H37 Ra. This was repeated 1 week later in the other flank. In addition, 400 ng of pertussis toxin diluted in PBS were injected intravenously on the day of immunization and repeated 48 h later. The mice were monitored daily following immunisation for weight loss and scored for neurological deficits using the same grading system as applied for assessment of acute EAE. Remissions were defined as improvement from the preceding relapse (or initial peak of disease) with a sustained (3 or more days) decrease in clinical score of at least 1.0. Relapses were identified by sustained (more than 3 days) increase of more than 1.0 clinical score from the baseline score of the previous remission.

All experiments were approved by the University of Oxford Clinical Medicine Ethical Review Committee and have been licensed under the Animals Scientific Procedures Act of the UK Home Office.

2.6.5. Isolation of murine microglia from adult mice

Mice were terminally anaesthetised using 100 µl of Pentobarbitone injected intraperitoneally and then perfused with cold PBS by cardiac puncture. Once perfusion with PBS was complete, the brain and spinal cord were collected in PBS on ice. These were placed in a dounce homogeniser containing 2 ml of the digestion cocktail [9.54 ml HBSS (Ca/Mg free, Sigma), 50 µl 10% collagenase D (0.05% final concentration, Roche), 10µl 100µg/ml Na-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (0.1µg/ml final concentration, Sigma) and 50µl of 5 U/ml DNase I (0.025 U/ml final concentration, Sigma)] and homogenised to make a cell suspension, with a loose fitting pestle. The cell suspension was then treated with an additional 8 ml of the digestion cocktail and rocked gently at room temperature for 15 min. This was allowed to settle for 15 min at room temperature and centrifuged for 7 min at 300 x g. The supernatant was discarded, pellet washed and re-suspended with 10 ml of HBSS (Sigma), followed by centrifuging for 7 min at 300 x g. The supernatant was once again discarded and cells re-suspended in 4 ml of 37% stock isotonic percoll (SIP, GE Healthcare) (prepared with 1x HBSS from a nine parts percoll with one part 10x PBS stock). This was then gradually layered over 4 ml of 70% SIP (prepared with 1x PBS from a nine parts percoll with one part 10x PBS stock), above which 4 ml of 30% SIP (prepared with 1x PBS from a nine parts percoll with one part 10x PBS stock) was overlaid, followed by 2 ml of 1X HBSS. The gradient was centrifuged for 40 min at 200 x g with minimal brake on the centrifuge. The cells at the 70-37% SIP interphase were collected and washed with 6 ml of 1X HBSS, by centrifuging for 7 min at 500 x g at 4ºC. These cells were then stained with anti-mouse CD45.2 PB (BD

Pharmigen) and anti-mouse CD11b APC (eBioscience) antibodies. The $CD11b^{+}CD45^{low}$ and $CD11b^{+}CD45^{high}$ populations were isolated by flow cytometry sorting with the help of Craig Waugh from the WIMM flow cytometry core facility and then used for RNA isolation.

2.7. Statistical analysis

Statistical analysis was performed using a two-tailed, unpaired Student's t-test. A P value less than 0.05 was used to indicate statistical significance. All statistical tests were performed using GraphPad Prism 5.0.

3. Results

3.1. Introduction

Microglial activation is crucial for the initiation and progression of MS and EAE, both by influencing the effector function of T cells and by directly causing tissue damage through innate immunity effector mechanisms (Gehrmann et al., 1993; Marik et al., 2007; Ponomarev et al., 2005; Rasmussen et al., 2007). Chronically activated microglia have been described to play an important role in the progressive phase of MS and CR-EAE by causing neuronal dysfunction, loss of neuronal synapses and axonal degeneration, which ultimately leads to permanent neurological disability (Kutzelnigg et al., 2005; Rasmussen et al., 2007; Zeis et al., 2008).

The exact cause of this persistent microglial activation has not been elucidated. Epigenetic mechanisms are known to maintain the gene expression profiles characteristic of differentiated cells and to regulate transcriptional changes in response to external cues (Jirtle and Skinner, 2007; Turner, 2007). Moreover, chromatin remodelling through histone modifications has been described to regulate the gene expression patterns of specific acquired immune responses (Wilson et al., 2009). Therefore, based on such observations, it was hypothesised that epigenetic-related changes could contribute to the initiation and maintenance of pathologically persistent microglial activation.

To date, only a single histone modifying enzyme, Jmjd3, a H3K27me3 demethylase, has been described to have a role in inflammation. While the role of Jmjd3 in regulating gene expression upon activation has already been characterised to a certain extent in macrophages (De Santa et al., 2009; De Santa et al., 2007; Ishii et al., 2009), it was not known if Jmjd3 or any other demethylase was involved in the activation process of microglia. Epigenetic changes are tissue and cell type specific, which means the same histone modifying enzyme could have

different roles in distinct cell types (Jaenisch and Bird, 2003). Therefore the findings obtained in macrophages could not be directly extrapolated to microglia. However, Jmjd3 up-regulation was shown to allow differentiated cells to reprogram their chromatin in response to environmental factors, providing a mechanism linking inflammation to the control of cell differentiation (De Santa et al., 2007; Ishii et al., 2009). This made it a good candidate for study in relation to chronic microglial activation and MS/EAE.

Given the important contribution of microglia to the CNS tissue injury in MS and EAE, this study aimed to identify epigenetic enzymes that could be involved in the maintenance of a gene expression program responsible for the chronic activation of microglia. To achieve this, the expression of *Jmjd3*, and other members of the JmjC domain protein family, was investigated in microglia activated with pro-inflammatory stimuli *in vitro*. The role of Jmjd3 in microglial activation was further explored by using ChIP-qPCR to identify potential target genes that could be involved in the induction and maintenance of this harmful chronic microglial activation. Subsequently, with the purpose of identifying a potential functional mechanism that could result in an increased transcription of such target genes, it was investigated if the binding of Jmjd3 following microglial activation had an influence on the H3K27me3 levels at their promoters.

3.2. Results

3.2.1. *Jmjd3* is up-regulated in activated microglia

De Santa *et al*. previously demonstrated that Jmjd3 is up-regulated and contributes to the control of gene expression in activated macrophages, following exposure to bacterial products and inflammatory cytokines (De Santa et al., 2007). Microglia are related to macrophages with respect to their myeloid origin and for being part of the mononuclear phagocyte system

(Kreutzberg, 1996). However, they are a distinctive cell type. Therefore, the involvement of Jmjd3 in regulating the activation process of microglia was undetermined. To initially examine the putative up-regulation of *Jmjd3* upon microglial activation, and to establish the appropriate stimulation conditions, such as the optimal LPS concentration, the EOC 20 murine microglial cell line was used. EOC 20 cells were stimulated with a titration of LPS, comparing treatment with 10, 100 and 1000 ng/ml for 2 hours. The mRNA level of *Utx*, a housekeeping H3K27me3 demethylase member of the same protein family as Jmjd3, but that is not inducible in differentiated cells (Agger et al., 2007; De Santa et al., 2007), was measured as a control.

The highest up-regulation of *Jmjd3* was observed with 100 ng/ml of LPS (24.5 fold induction). *Utx* was not up-regulated upon LPS stimulation, as expected, and instead showed a consistently low-level expression compared to *Jmjd3* (Figure 3.1).

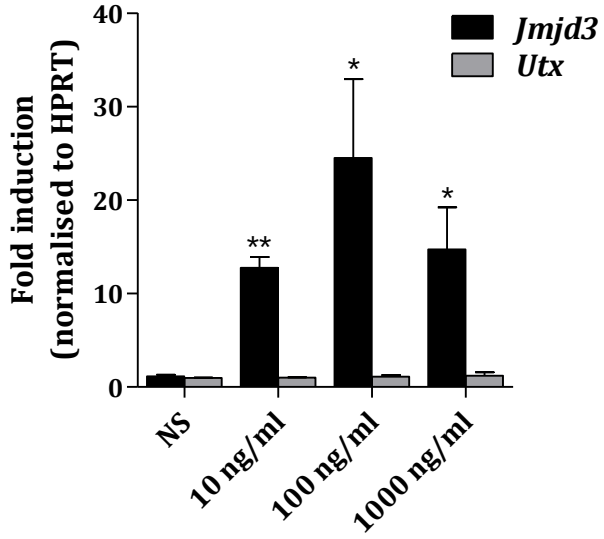


Figure 3.1. *Jmjd3* is up-regulated in LPS-treated EOC 20 cells. EOC 20 cells were stimulated for 2 hours with a titration of LPS. The mRNA levels of *Jmjd3* and *Utx* were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). Representative data from 2 experiments. Bars represent mean ± standard error between triplicates. *$p < 0.05$. **$p < 0.01$.

3.2.2. Induction of JmjC-domain containing proteins by pro-inflammatory stimuli in primary murine microglia

Having determined that *Jmjd3* expression is increased by LPS in a microglial cell line and having established the optimal concentration to achieve this up-regulation, only primary cells were used for further gene expression experiments. This was done to avoid possible bias in the epigenetic profile introduced by immortalisation in the cell line. For example, it has been demonstrated that in lymphoblastoid cell lines and induced pluripotent and human ES cell lines the DNA methylation patterns are different from those of their primary counterparts (Grafodatskaya et al., 2010; Lister et al., 2011; Sugawara et al., 2011).

Since Jmjd3 is a JmjC-domain containing protein, other demethylases which are part of this group could also potentially be involved in the regulation of transcription in activated microglia. Therefore it was investigated if their expression was also affected by LPS treatment. A qRT-PCR screen for all the JmjC-domain family proteins, including *Jmjd3*, was performed in primary murine microglia cultured from inbred wild-type C57BL/6 mice pups.

The primary microglial cells were stimulated with 100 ng/ml of LPS for 2, 4, 8 and 24 hours. This LPS concentration was chosen because it produced the highest *Jmjd3* up-regulation in the microglial cell line and it is the same concentration used by De Santa et al. for the analogous experiment with macrophages (De Santa et al., 2007). Furthermore, this concentration has also been previously described to result in primary microglial cell activation (Lund et al., 2006; Olson and Miller, 2004; Schmid et al., 2009).

Of the 25 genes analysed, *Jmjd3* and *Jarid2* were the only proteins that showed significant mRNA up-regulation upon LPS stimulation, with *Jmjd3* being the most strongly induced (20.34 fold induction after 2 hours) (Figure 3.2).

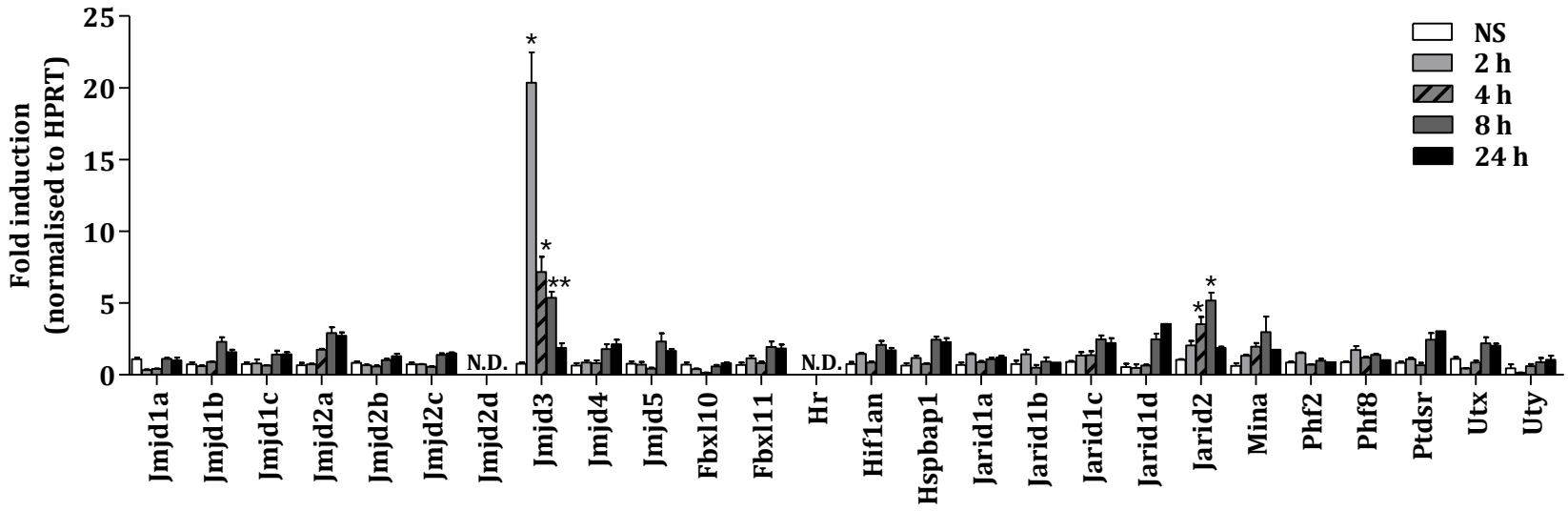


Figure 3.2. Expression of JmjC-domain containing proteins in LPS-treated primary microglia. Cells were incubated with 100 ng/ml of LPS for the indicated time periods. The mRNA levels of JmjC-domain proteins were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). N.D. indicates not detected. Bars represent mean ± standard error between triplicates. *p >0.05. **p < 0.01.

Inflammation in MS and EAE is associated with an IFNγ-producing T_H1 response and the cytokine is found at high levels in active MS lesions (Cannella and Raine, 1995; Olsson et al., 1990). IFNγ is also known to activate microglia and differentiate them in to APCs which release pro-inflammatory cytokines. Therefore, due to this being more physiologically relevant in both MS and EAE, the effect of IFNγ on the mRNA expression of JmjC-domain containing proteins in primary microglia was also investigated. Primary murine microglia were treated with 100 U/ml of IFNγ for 2, 4 and 8 hours. This concentration was chosen because it has consistently been used in previous studies to stimulate microglial cells (Moran et al., 2007; Moran et al., 2004; Olson et al., 2001).

Jmjd3 was the only JmjC protein with a significant increase in gene expression after IFNγ treatment. However, this up-regulation was lower than when stimulating with LPS, as shown previously: 7.4 fold induction with IFNγ (Figure 3.3) in contrast to 20.3 fold induction after 2 hours incubation with LPS (Figure 3.2).

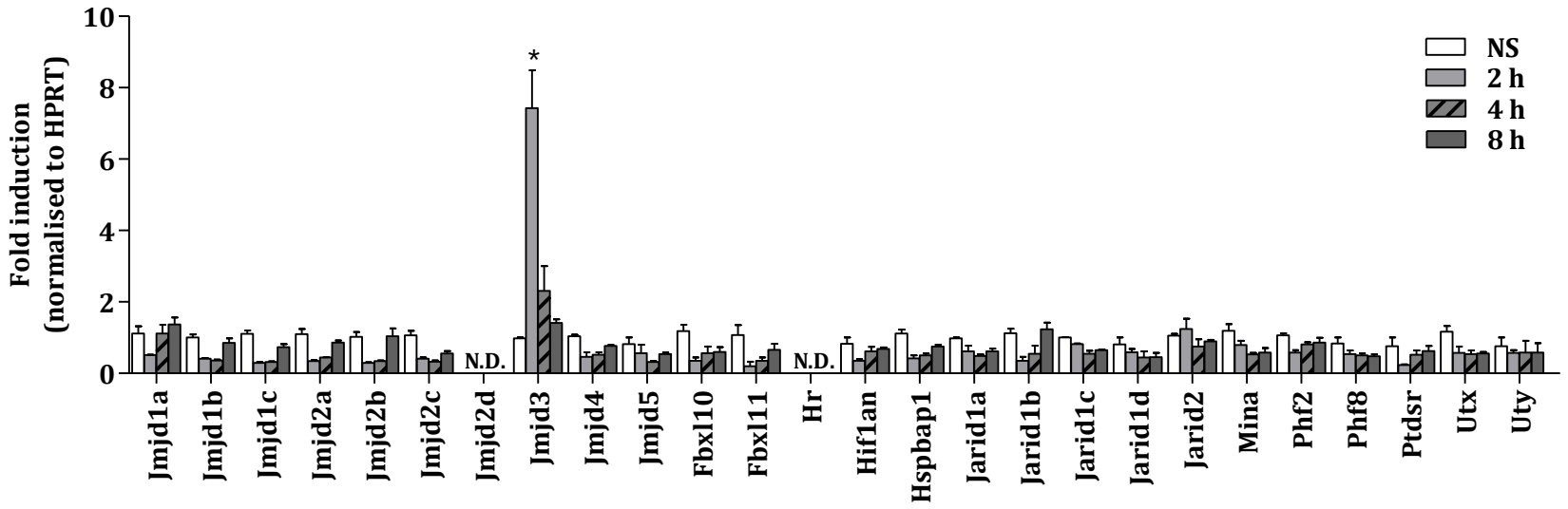


Figure 3.3. Expression of JmjC-domain proteins in IFNγ-treated primary microglia. Cells were incubated with 100 U/ml of IFNγ for 0, 2, 4 and 8 hours. The mRNA levels of JmjC-domain proteins were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). N.D. indicates not detected. Bars represent mean ± standard error between triplicates. *p >0.05.

3.2.3. *Jmjd3* up-regulation in activated primary microglia

Since *Jmjd3* was the only JmjC protein induced by IFNγ stimulation and was considerably more up-regulated by LPS than *Jarid2* in primary microglia, *Jmjd3* was selected for further characterisation of its expression upon activation. The selection of Jmjd3 was also based on the fact that its role in LPS plus IFNγ-activated macrophages has been described previously (De Santa et al., 2007). Additionally, Jarid2 was not further investigated because it has been shown to only play a major role during early development, when it regulates the binding of the H3K27 methyltransferase complex PRC2 to target genes in ES cells to promote differentiation (Pasini et al., 2010), and therefore was considered less relevant in the context of increasing understanding of MS pathogenesis.

To better characterise the kinetics of *Jmjd3* up-regulation and to establish whether the previously examined 2 hour time point is the point at which *Jmjd3* is increased the most, a time course experiment was conducted. Primary murine microglial cells were treated with 100 ng/ml of LPS for 30 minutes, 1, 2, 4, 8 and 24 hours. *Utx* was, again, included in the qRT-PCR analysis as a negative control.

Jmjd3 mRNA levels were more strongly induced after 1 hour stimulation with LPS (31.9 fold induction), decreasing to less than half after 4 hours (12.2 fold induction) and returning close to basal levels after 24 hours stimulation (3.6 fold induction). *Utx* expression remained at a low, constant level, as expected (Figure 3.4).

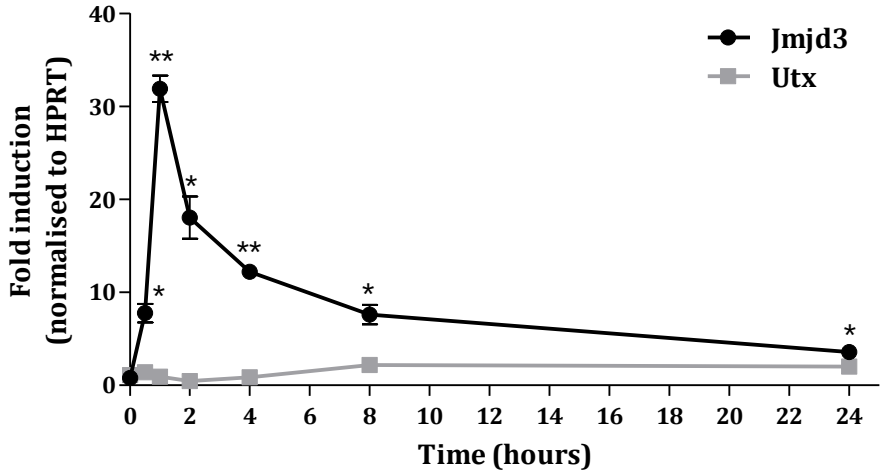


Figure 3.4. Kinetics of *Jmjd3* mRNA up-regulation in primary microglia. Cells were treated with 100 ng/ml of LPS for the indicated time points. The mRNA levels of *Jmjd3* and *Utx* were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated. Data are represented as mean ± standard error between triplicates. *p < 0.05. **p < 0.01.

TLRs 2, 3, 4 and 9 have previously been found to be up-regulated in MS and EAE lesions and stimulation of "resting" microglia through these receptors leads to a pro-inflammatory activated phenotype *in vitro* (Bsibsi et al., 2002; Olson and Miller, 2004; Prinz et al., 2006). Moreover, it has been described that certain viral and bacterial infections can exacerbate and even initiate MS (Andersen et al., 1993; Kurtzke, 1993; Wolfson and Talbot, 2002) and TLRs can also be activated by endogenous ligands released after tissue injury without the presence of pathogens (Zhang and Schluesener, 2006). Hence, triggering microglial activation by TLR engagement might play an important role in the pathogenesis of MS and EAE. Therefore the impact of TLR agonists (other than LPS) on *Jmjd3* expression in primary microglia was investigated. This with the objective to generate a hypothesis as to which types of infections or inflammatory stimuli can occur in the CNS of MS patients that could be stimulating Jmjd3 up-regulation in microglia through TLR signalling.

Primary microglia were treated with the TLR1/2 agonist Pam3CSK4, the TLR3 agonist Poly (I:C), the TLR 2/6 agonist FSL-1, the TLR7 agonist Imiquinoid and the TLR9 agonist CpG oligonucleotide. The concentrations used were chosen according to the supplier's suggestions and also previous literature (Olson and Miller, 2004). The incubation time of 1 hour was selected as this corresponds to the peak of *Jmjd3* up-regulation observed when stimulating with the TLR4 agonist, LPS (Figure 3.4).

As shown in figure 3.5, all of the TLR agonists tested induced *Jmjd3* expression, with LPS being, again, the strongest stimulus, followed by FSL-1 (TLR 6/2), Pam3CSK4 (TLR 1/2), Imiquinoid (TLR7), Poly (I:C) (TLR3) and finally CpG oligonucleotide (TLR9).

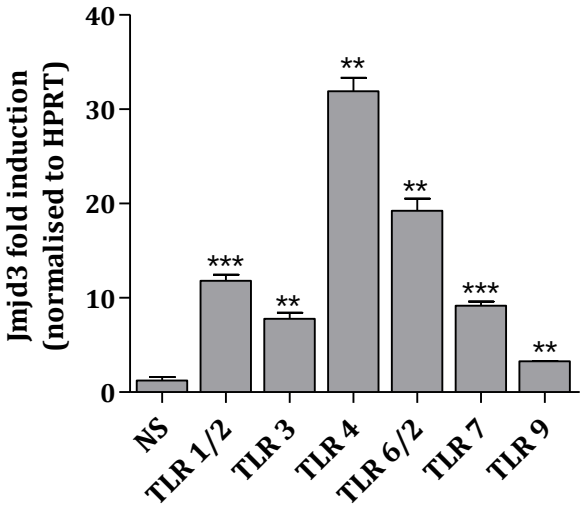


Figure 3.5. *Jmjd3* expression in primary microglia following TLR stimulation. Cells were treated for 1 hour with different TLR agonists. TLR 1/2: 1µg/µl of Pam3CSK4, TLR 3: 25 µg/µl of Poly (I:C), TLR 6/2: 1 µg/µl of FSL-1, TLR 7: 10 ug/ml of Imiquimod, and TLR 9: 2µM of CpG ODN1826. The mRNA level of *Jmjd3* was measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). Bars represent mean ± standard error between triplicates. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

Subsequently, it was investigated if other pro-inflammatory cytokines and chemokines that have been previously described to be relevant to the MS lesion pathology have an influence on microglial *Jmjd3* expression.

IL-1β and IL-8 (CXCL-8) are elevated in the CSF of patients with MS (Bartosik-Psujek et al., 2004; Deckert-Schluter et al., 1992; Ishizu et al., 2005), and IL-1β has also been found at high levels within demyelinated lesions (Hofman et al., 1986; McGuinness et al., 1997). Microglia are the main source of IL-1β resulting from CNS trauma, infection and inflammation (Brenneman et al., 1992; Eriksson et al., 1999). However, neurons, astrocytes, oligodendrocytes, infiltrating monocytes and endothelial cells in the CNS can also produce IL-1β under such pathological conditions (Blasi et al., 1999; Davies et al., 1999; Pearson et al., 1999). Importantly, microglia also express the IL-1 receptor (Pinteaux et al., 2002), which enable them to respond to IL-1β in an autocrine, as well as in a paracrine, manner. This establishes a positive feedback loop that stimulates further microglial activation and amplifies the neuroinflammatory process. IL-1β-activated microglial cells have been shown to produce TNFα, IL-1β, IL-6, IL-8, CCL2, CCL3, CCL4, CCL5 and NO (Basu et al., 2002a; Basu et al., 2002b; McManus et al., 1998) which are all important pro-inflammatory and immunoregulatory mediators in MS.

The chemokine IL-8 is also produced by a wide variety of cells, including endothelial cells, monocytes, T cells and activated microglia (Ehrlich et al., 1998; Prat et al., 2002; Schroder et al., 1987; Wechsler et al., 1994). In MS, IL-8 plays a major role in mediating the recruitment of monocytes to the CNS (Gerszten et al., 1999; Lund et al., 2004). Apart from its chemo-attractant properties, IL-8 has been shown to discretely induce the expression of IL-6, TNFα, IL-1β and IL-8 in microglia, and perhaps more importantly it seems to enhance the effect of other more potent stimuli in the microglial production of pro-inflammatory agents (Franciosi et al., 2005).

Given the important autocrine and paracrine roles of IL-1β and IL-8 in microglial activation and MS pathogenesis, the following experiment was performed. Primary microglia were incubated with 100 U/ml of IL-1β and 100 ng/ml of IL-8 for 1 hour (peak of *Jmjd3* up-regulation after LPS treatment). These concentrations were chosen because they were used in previous reports to stimulate microglia (Cross and Woodroofe, 1999; Ehrlich et al., 1998; Franciosi et al., 2005; Lee et al., 1993).

Jmjd3 expression was significantly increased by both IL-1β and IL-8, with IL-1β being the strongest stimulant of the two (Figure 3.6). Although this experiment was not run in parallel with the previous TLR agonists stimulation assays, the methodology used was the same. The difference between the fold induction values obtained in these two experiments suggest that most of the TLR agonists, especially LPS, are more potent inducers of *Jmjd3 than* IL-1β and IL-8. This is interesting because it reflects the previously described versatility of microglial responses to different activating conditions (Butovsky et al., 2006a; Butovsky et al., 2006b; Hanisch and Kettenmann, 2007; Magnus et al., 2001). While TLR activation of microglia by infectious agents produces strong defense reactions that release neurotoxic factors (Dalpke et al., 2002; Lehnardt et al., 2003; Olson and Miller, 2004), and in this case induce higher levels of *Jmjd3*, minor insults accompanied by a moderate production of one or few cytokines/chemokines (such as the damage of a synapse or the apoptosis of a few neurons) induce more contained responses that promote tissue repair and neuronal/glial survival (Butovsky et al., 2006b; Magnus et al., 2001; Trapp et al., 2007).

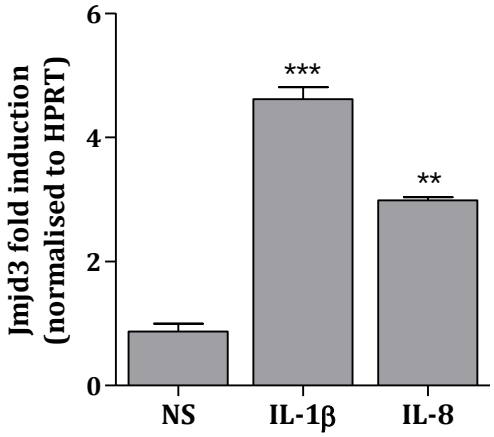


Figure 3.6. *Jmjd3* expression in primary microglia following stimulation with IL-1β and IL-8. Cells were incubated with 100 U/ml of IL-1β and 100 ng/ml of IL-8 for 1 hour. The mRNA level of *Jmjd3* was measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). Bars represent mean ± standard error between triplicates. **p < 0.01. ***p < 0.001.

IFNγ plays a central part in MS and EAE by activating microglia to become APCs and to secrete cytotoxic factors as well as cytokines that induce naïve $CD4^+$ T cells to differentiate into IFNγ-producing T_H1 cells, perpetuating inflammation in the CNS. Given that IFNγ has such an important role in MS and EAE inflammatory processes, the IFNγ-induced *Jmjd3* up-regulation profile was examined using a greater range of time points. Primary murine microglial cells were treated with 100 U/ml of IFNγ for 1, 2, 4, 8 and 24 hours.

The IFNγ-induced *Jmjd3* kinetics curve showed the same shape as the LPS one, but with the cytokine producing lower expression values overall. The highest *Jmjd3* mRNA up-regulation was 10.1 fold induction after 1 hour of IFNγ stimulation, in contrast to 31.9 fold induction with LPS, when compared to non-stimulated cells (Figure 3.7).

To evaluate whether multiple stimuli had a greater ability to stimulate primary microglia in comparison to stimuli given individually, primary microglial cells were treated with 100

ng/ml of LPS plus 100 U/ml of IFNγ for 1, 2, 4, 8 and 24 hours. The combination of these two specific stimulants was chosen because it has been demonstrated that this cytokine primes macrophages for more rapid and stronger responses to LPS (Pace et al., 1983).

Although treatment with LPS alone gave a higher *Jmjd3* mRNA level after 1 hour stimulation, the addition of both LPS and IFNγ produced a higher level of expression after 2 and 4 hours incubations (Figure 3.7). To ensure that optimal amounts of protein were produced for further characterisation, and in line with the work performed previously by other groups studying the role of Jmjd3 in macrophages (De Santa et al., 2009; De Santa et al., 2007), stimulation with LPS plus IFNγ for 2 hours was used in successive assays.

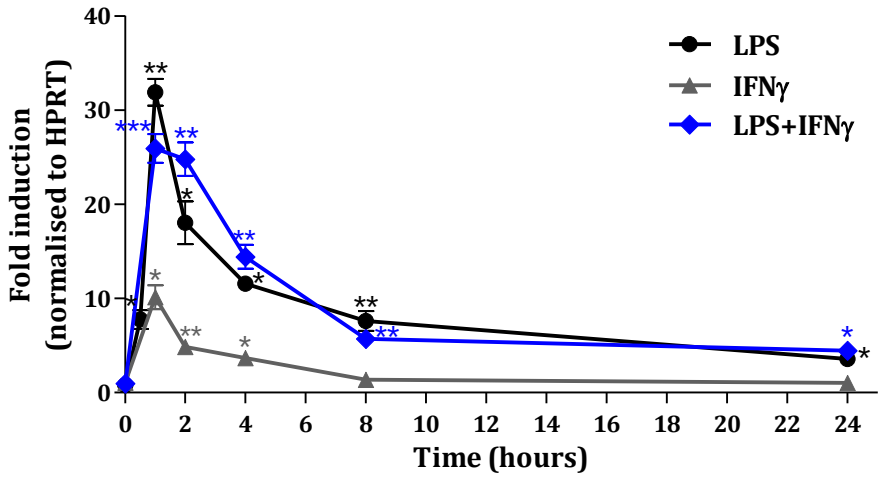


Figure 3.7. Kinetics of *Jmjd3* mRNA up-regulation in primary microglia after treatment with LPS and IFNγ alone or in combination. Cells were treated with 100 ng/ml LPS, 100 U/ml IFNγ and 100 ng/ml LPS plus 100 U/ml IFNγ for the indicated time points. The mRNA level of *Jmjd3* was measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated. Data are represented as mean ± standard error between triplicates. *p < 0.05. **p < 0.01. ***p < 0.001.

Next, to confirm that the up-regulation observed at the mRNA level was translated to protein, the protein level Jmjd3 expression was analysed in activated primary murine microglia by

immunocytochemistry. The cell cultures were either left untreated or stimulated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2 and 4 hours. The cells were also immunostained for CD11b and counterstained with the nuclear dye DAPI. CD11b, or integrin alpha M chain is widely used as a membrane microglial marker. It combines with the beta 2 chain (ITGB2) to form the integrin known as macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3) (Ford et al., 1995; Sedgwick et al., 1991).

Non stimulated primary microglia showed a ramified morphology and low expression of Jmjd3 in the nucleus (16.69% of the quantified nuclei were positive for Jmjd3) (Figure 3.8A and 3.8D). After treatment with 100 ng/ml of LPS plus 100 U/ml of IFNγ, microglia adopted a rounder, ameboid shape and the levels of Jmjd3 in the nucleus increased, peaking after 2 hours incubation (42.38%) (Figure 3.8B and 3.8D). After 4 hours treatment, Jmjd3 protein expression decreased to 27.15%, but was still higher than in non-activated microglia (Figure 3.8C and 3.8D). Low Jmjd3 expression was also detected in the cytoplasm. This could correspond to protein being synthesised and processed in the endoplasmic reticulum and Golgi apparatus, especially since it was detected adjacent to the nucleus. However, detection of Jmjd3 outside of the nucleus was not included in the quantification.

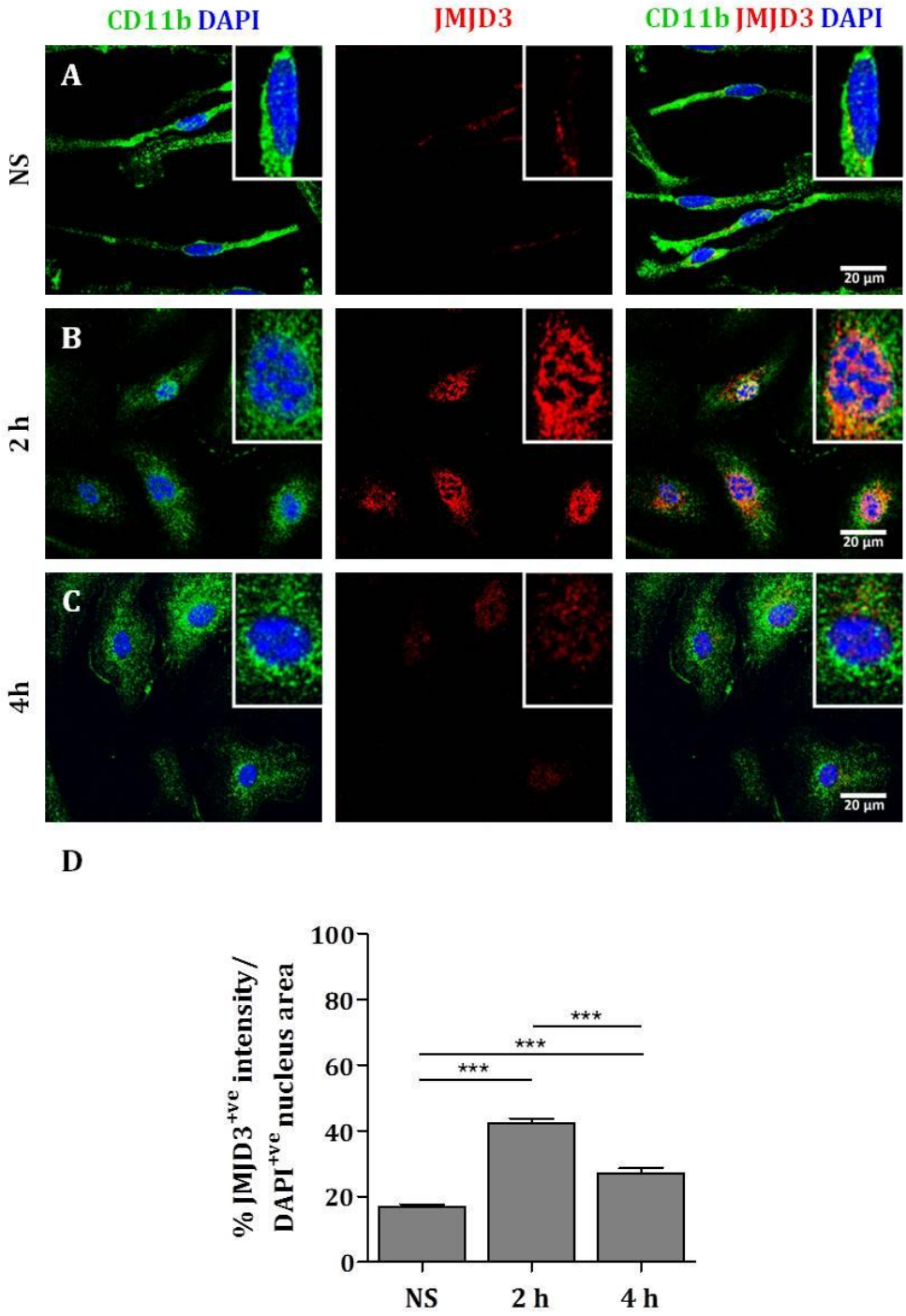


Figure 3.8. Jmjd3 induction at the protein level in LPS+IFNγ-treated primary microglia. (A-C) Microglial cultures were either (A) non stimulated (NS), or stimulated for (B) 2 or (C) 4 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ. Cells were then immunostained for immunohistochemical analysis with antibodies for the membrane marker Cd11b and Jmjd3, and counterstained with the nucleic acid stain DAPI. Representative images are shown. (D) Quantification of Jmjd3 positive nuclei calculated by dividing the % of average positive Jmjd3 intensity by the DAPI positive area. 200-250 nuclei were quantified per condition. Bars represent mean ± standard error. ***$p < 0.001$.

Given that Jmjd3 is a H3K27me3 demethylase (Agger et al., 2007; Hong et al., 2007; Xiang et al., 2007), it was investigated whether the observed Jmjd3 up-regulation, both at the mRNA and protein levels, was accompanied by cell-wide changes in the histone methylation of H3K27. The levels of the histone mark were examined by immunocytochemistry. Both unstimulated and LPS plus IFNγ-stimulated primary microglia were stained with anti-CD11b and anti-H3K27me3 antibodies and counterstained with DAPI.

Jmjd3 induction in LPS plus IFNγ-activated microglia did not cause any detectable reduction in H3K27me3 after 2 and 4 hours incubations (Figure 3.9).

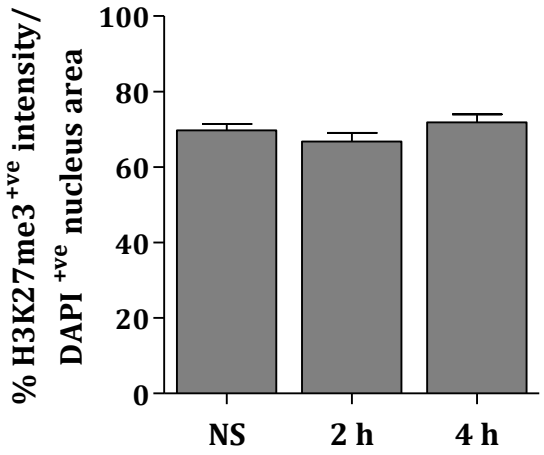


Figure 3.9. Global H3K27me3 levels in LPS+IFNγ-treated primary microglia. Microglial cultures were either non stimulated, or stimulated for 2 or 4 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ. Cells were then immunostained for immunohistochemical analysis with antibodies for the membrane marker Cd11b and the histone mark H3K27me3, and counterstained with the nucleic acid stain DAPI. The H3K27me3 levels remained the same as in the ramified, non stimulated (NS) microglia after activation with 100 ng/ml of LPS plus 100 U/ml of IFNγ. The graph shows the quantification of H3K27me3 positive nuclei calculated by dividing the % of average positive H3K27me3 intensity by the DAPI positive area. 200-250 nuclei were quantified per condition. Bars represent mean ± standard error.

The levels of H3K4me3, an epigenetic mark for transcription activation (Kouzarides, 2007), were also tested. Similarly, no global changes in H3K4me3 were observed (Figure 3.10).

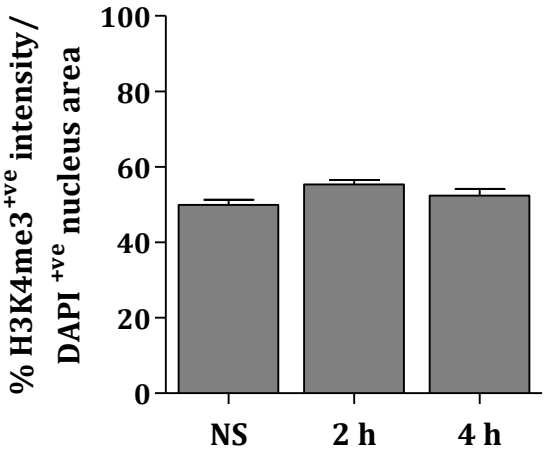


Figure 3.10. Global H3K4me3 levels in LPS+IFNγ-treated primary microglia. Microglial cultures were either non stimulated, or stimulated for 2 or 4 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ. Cells were then immunostained for immunohistochemical analysis with antibodies for the membrane marker Cd11b and the histone mark H3K4me3, and counterstained with the nucleic acid stain DAPI. The H3K4me3 levels remained the same as in the ramified, non stimulated microglia after activation with 100 ng/ml of LPS plus 100 U/ml of IFNγ. The graph shows the quantification of H3K4me3 positive nuclei calculated by dividing the % of average positive H3K4me3 intensity by the DAPI positive area. 200-250 nuclei were quantified per condition. Bars represent mean ± standard error.

3.2.4. Expression of immune response and inflammation related genes in activated primary microglia

To identify genes in microglia which are up-regulated upon activation and that therefore could potentially be regulated by Jmjd3, the expression of a group of inflammation-related, inducible genes was studied, including *Ccl3*, *Ccl5*, *Ccl9*, *Cxcl11*, *Il6*, *Il12b*, *Nos2* and *TNFα*. These genes were selected because of their relevance to the MS/EAE immunopathogenesis and also because in *Jmjd3*$^{-/-}$ macrophages, *Ccl3*, *Ccl5*, *Ccl9*, *Cxcl11*, *Il12b* and *Il6* have previously been described to be partially dependent on Jmjd3 for expression (De Santa et al., 2009). *Nos2* and *TNFα* do not seem to be differentially expressed in *Jmjd3*$^{-/-}$ macrophages. However, the *Nos2* promoter shows important Jmjd3 binding in LPS plus IFNγ activated mouse macrophages and *TNFα* is also bound by Jmjd3 upstream of its promoter in such cells. NO and TNFα were

additionally included in the analysis because they are important soluble mediators of the inflammatory innate immune response that damages the oligodendrocyte/myelin unit and axons in MS and EAE. Primary microglial cultures were treated with LPS plus IFNγ and the mRNA levels of the selected genes were measured by qRT-PCR. The kinetics of their up-regulation were determined by using different time periods of stimulation.

All the examined genes were significantly up-regulated at the mRNA level after 1 hour treatment. With the exception of *TNFα*, whose mRNA level peaked after 4 hours, the highest expression with all other genes was reached following 8 hours stimulation (Figure 3.11).

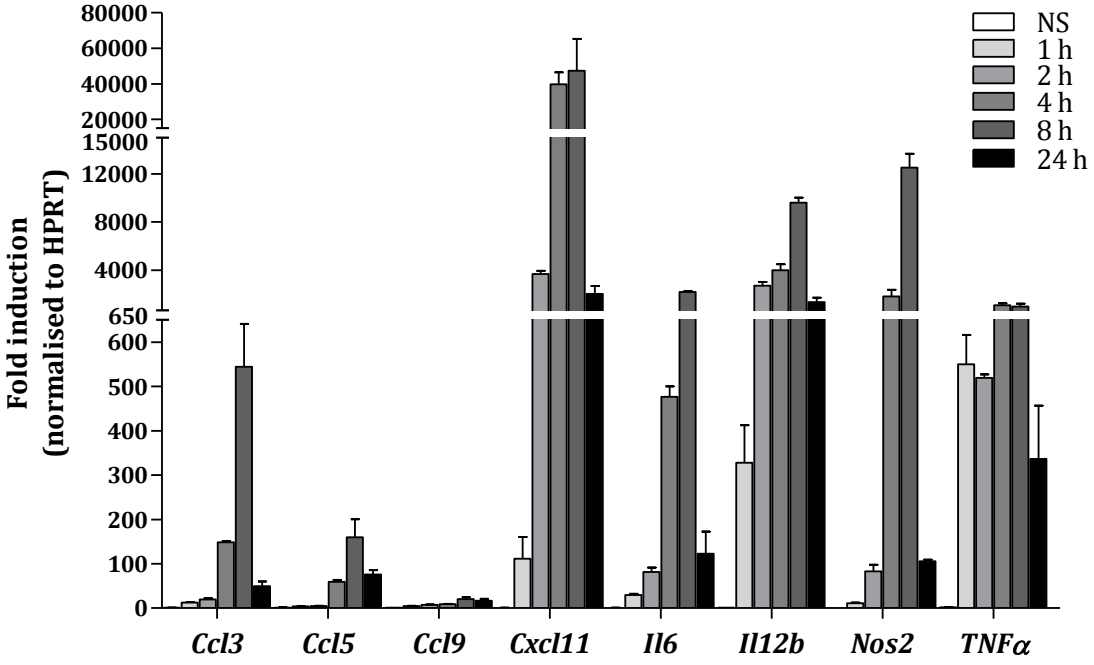


Figure 3.11. Immune response and inflammation related genes up-regulated upon activation in primary microglia. Microglial cells were incubated with 100 ng/ml LPS plus 100 U/ml of IFNγ for the indicated periods of time. The mRNA levels were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non stimulated (NS). Bars represent mean ± standard error between triplicates. All the genes were significantly up-regulated above background levels with a p value < 0.05.

3.2.5. Jmjd3 binds to the promoter of immune response and inflammation related genes in activated microglia

While the effects of Jmjd3 up-regulation on LPS plus IFNγ activated macrophages have been reported to be independent of its H3K27me3 demethylase activity (De Santa et al., 2009), for alternative activated macrophages the opposite has been shown (Ishii et al., 2009). ChIP assays for Jmjd3 and H3K27me3 were used to investigate if Jmjd3 up-regulation in activated microglia was followed by changes in the level of H3K27me3, as a putative mechanism to control gene expression. ChIP-qPCR experiments were performed initially with the expectation of carrying out subsequent ChIP-sequencing analysis depending on the results obtained with this and further functional experiments.

The lack of global changes in H3K27me3 following the rapid and strong up-regulation of Jmjd3 observed in microglia after exposure to inflammatory stimuli, shown previously (Figure 3.9), suggested that if Jmjd3 was acting as a H3K27me3 demethylase in activated microglia, it would only be at specific gene promoters. Therefore, six of the inducible immune response and inflammation related genes, which expression kinetics were analysed previously in activated microglia (Figure 3.11), were selected for the Jmjd3 and H3K27me3 ChIP-qPCR analysis. With the exception of *Nos2,* only genes which have been previously shown to be regulated by Jmjd3 in macrophages were investigated because of the high number of cells required to perform these assays, and the low yield of microglia which can be obtained per pup.

In order to first establish suitable parameters for the assay, the EOC 20 microglial cell line was used before moving to primary cells. EOC 20 cells were either left untreated or stimulated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2 hours, corresponding to the peak of Jmjd3 up-regulation at the protein level, as shown previously (Figure 3.8). The recruitment of Jmjd3 to the promoter regions of *Ccl3*, *Ccl5*, *Cxcl11*, *Il6*, *Il12b* and *Nos2* was analysed by conducting

qPCRs on the immunoprecipitated chromatin. The promoter of guanylate-binding protein 6 (*Gbp6*) and the TSS1 of *Arhgef3* were included in the study as positive and negative controls, respectively, as used previously in activated macrophage studies (De Santa et al., 2009).

This preliminary assay suggested that in EOC 20 microglia, Jmjd3 was associated to the promoters of *Ccl3*, *Ccl5*, *Nos2* and the positive control *Gbp6* when treated with LPS plus IFNγ compared to untreated cells. However *Il12b* and *Il6*, also previously shown to be bound by Jmjd3 in activated primary macrophages (De Santa et al., 2009), showed no Jmjd3 enrichment (Figure 3.12).

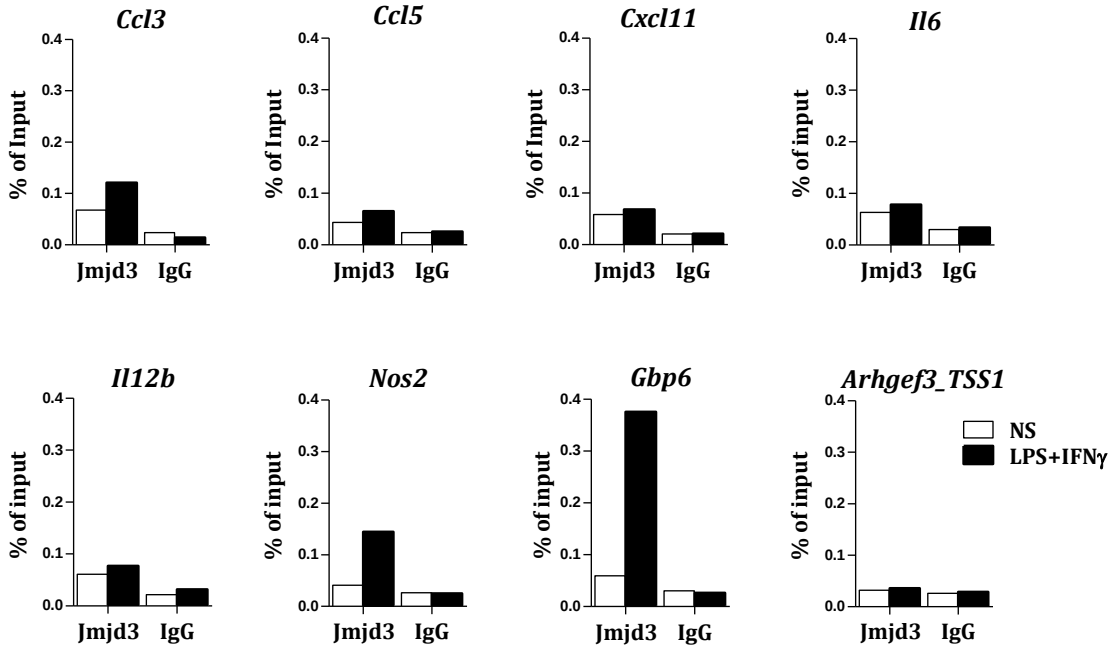


Figure 3.12. Jmjd3 binds to the promoter of immune response and inflammation related genes in LPS+IFNγ-activated EOC 20 microglia. An anti-Jmjd3 ChIP assay was performed in EOC 20 cells treated for 2 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ and in non stimulated (NS) cells. Immunoprecipitated DNA with an anti-Jmjd3 antibody or an anti-IgG isotype control was amplified by qPCR with promoter-specific primers. The chromatin Jmjd3 enrichment was calculated as percentage (%) of input. *Gbp6* was used as positive region control and *Arhgef3_TSS1* as a negative region control.

After standardising the assay in EOC 20 cells, the recruitment of Jmjd3 to the same group of genes was examined by ChIP-qPCR in primary microglial cells. As before, non-stimulated cells and cells treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2 hours were compared. Jmjd3 was significantly enriched in the promoter region of *Ccl3*, *Ccl5*, *Il6*, *Nos2* and *Gbp6* (positive control) in the activated primary microglia. This coincides with the preliminary results obtained with the cell line, except that Jmjd3 also binds the promoter of *Il6* after stimulation in primary microglia. Once again, no enrichment of Jmjd3 was observed at the *Il12b* region examined (Figure 3.13).

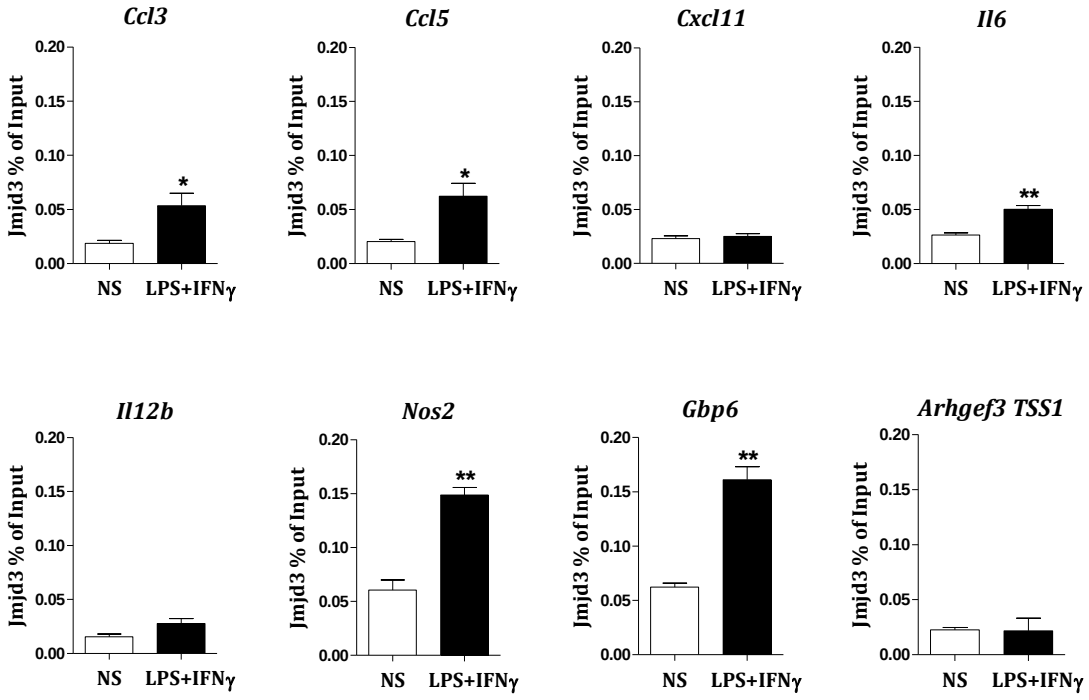


Figure 3.13. Immune response and inflammation related genes are targets of Jmjd3 in LPS+IFNγ-activated primary microglia. An anti-Jmjd3 ChIP assay was performed in primary murine microglia treated for 2 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ and in non stimulated cells (NS). Immunoprecipitated DNA with an anti-Jmjd3 antibody was amplified by qPCR with promoter-specific primers. *Gbp6* was used as positive region control and *Arhgef3_TSS1* as a negative region control. Bars represent mean ± standard error between triplicates. *p < 0.05. **p < 0.01.

3.2.6. H3K27me3 status at Jmjd3 target genes

To test whether the observed binding of Jmjd3 to the promoter region of *Ccl3*, *Ccl5*, *Il6* and *Nos2* in activated primary microglia correlated with changes in the H3K27me3 levels, a ChIP-qPCR analysis of the chromatin modification was performed.

All the gene promoter regions analysed, except *Arhgef3_TSS1,* showed a significant reduction in the levels of H3K27me3 after LPS plus IFNγ treatment compared to no stimulation (Figure 3.14). No controls for active or repressed chromatin were included because of sample quantity limitations caused by the low number of primary microglia obtainable per pup. However the fact that *Gbp6* showed a marked reduction in H3K27me3 could be considered as an indication that the assay worked because *Gbp6* is a gene that has been described as highly induced by LPS and IFNγ (De Santa et al., 2009) and is therefore transcriptionally active after stimulation of microglia with these agonists. Similarly, because the transcription of *Arhgef3* usually starts at the TSS2 or TSS3 (Thiesen et al., 2000), the lack of significant changes in the H3K27 trimethylation at the TSS1, which is a region that is not important for activation of transcription, could be regarded as a negative control.

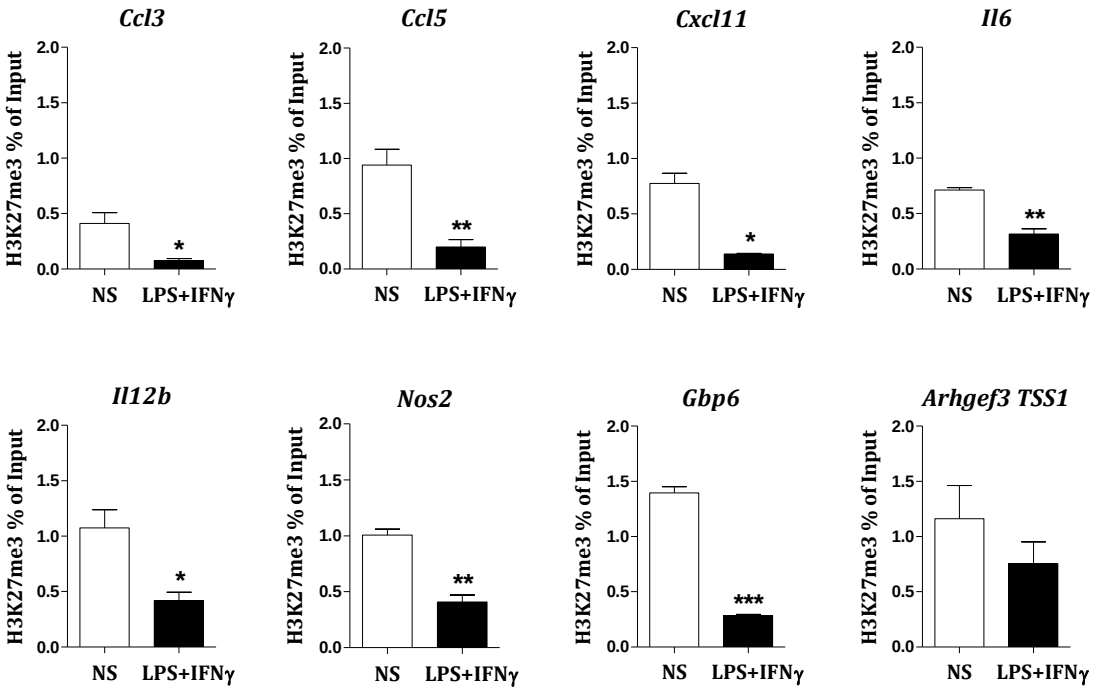


Figure 3.14. H3K27me3 levels at immune response and inflammation related genes in LPS+IFNγ-activated primary microglia. An anti-H3K27me3 ChIP assay was performed in primary murine microglia treated for 2 hours with 100 ng/ml of LPS plus 100 U/ml of IFNγ and in non stimulated cells (NS). Immunoprecipitated DNA was amplified by qRT-PCR with promoter-specific primers. Data are representative of 3 independent experiments. Bars represent mean ± standard error. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

3.3. Discussion

The qRT-PCR experiments with the EOC 20 microglial cell line and the primary murine microglia showed that Jmjd3 is up-regulated after exposure to LPS. This agrees with the hypothesis that changes in the expression of Jmjd3 can influence the transcription of genes that contribute towards pathological persistent activation of microglia upon stimulation with pro-inflammatory stimuli. With the exception of Jmjd3, there has been no previously described role for the JmjC-domain demethylases with regards to the immune response or inflammation. Jmjd3 is a NF-κB-dependent H3K27me3 demethylase (Agger et al., 2007; Xiang et al., 2007) that is induced in activated macrophages and regulates transcription (De Santa et

al., 2009). Given their early myeloid origin, microglial cells are considered part of the mononuclear phagocyte system and are therefore related to macrophages (Ginhoux et al., 2010; Kreutzberg, 1996). However, microglia are a distinct cell type and it cannot be assumed that Jmjd3 has the same role in microglia as it does in macrophages. Therefore the involvement of Jmjd3 in regulating the activation transcriptional program of microglia was previously undetermined.

The gene activation by NF-kB proteins is regulated by several complex mechanisms that include the control of the rate of transcription induction. In this way, even if the stimulus is constant, all of the NF-kB-dependent genes are not activated at the same time. The speed of gene induction by NF-kB is determined by the DNA and chromatin structure of the NF-kB target genes' control regions. This dictates if other constitutive or inducible transcription factors and transcriptional co-regulators (such as histone modifying enzymes and nucleosome remodelling complexes) are also required to activate gene expression (Natoli et al., 2005; Sen and Smale, 2010). *In vitro* experiments showed that Jmjd3 was basally expressed in non-stimulated cells and then strongly and rapidly up-regulated at the mRNA and protein levels after activation with LPS, as observed in macrophages. On the other hand, the transcript levels of *Utx*, a H3K27me3 demethylase known to have a role in development rather than in inflammation (Agger et al., 2007; De Santa et al., 2007), remained low and unaltered upon stimulation. The kinetics of *Jmjd3* up-regulation in activated microglia shows that it is a rapidly induced NF-kB target gene. This suggests that Jmjd3 might act as a transcription co-regulator of other genes that are subsequently induced by NF-κB after microglial activation, whose promoters require other earlier induced factors apart from NF-κB to be activated. This is further supported by Hao *et al.'s* observations on the expression patterns of TNFα inducible genes. They described that genes induced during an inflammatory response can be divided in three groups according to their expression kinetics, and that genes within each group show

similar or related functions. Transcription factors and modulators fall within the early induced genes group, whose expression peaked before 2 hours stimulation and had short half-lives (Hao and Baltimore, 2009). Since *Jmjd3* expression peaked at 1 hour, it can be assigned to this group and could therefore be a transcription modulator of intermediate and late genes such as most cytokines and chemokines.

Interestingly, stimulation with 1000 ng/ml of LPS produced a lower *Jmjd3* induction compared to 100 ng/ml in EOC 20 cells. This could be explained by increased receptor internalisation after exposure to a higher LPS concentration. Nomura *et al.* first described that the TLR4 mRNA and surface protein expression levels were reduced after 1 hour stimulation with 100 ng/ml of LPS in peritoneal macrophages, and that such reduction was increased by higher LPS concentrations (Nomura et al., 2000). It was later shown that the reduction in the amount of TLR4 receptor on the cell surface was secondary to receptor internalisation. TLR4 endocytosis after microbial detection was first thought to be a regulatory mechanism for signal termination and LPS-associated antigen presentation (Husebye et al., 2006). However, it was later found that the TLR4 internalisation into the endosomal compartment is necessary for triggering the MyD88-independent signalling pathway through the adaptors TRAM and TRIF, which activate the transcription factor IRF3 that regulate type I interferon expression (Kagan et al., 2008). Indeed, in mouse bone marrow derived macrophages the percentage of surface TLR4 starts decreasing after only 10 minutes of LPS stimulation and the expression goes down to around 50% after 30 minutes treatment (Zanoni et al., 2011).

It was important to investigate the role of TLR ligands on *Jmjd3* expression because there is a marked up-regulation of all TLR receptors in MS lesions and EAE (Bsibsi et al., 2002; Prinz et al., 2006) and the latter is induced with an adjuvant containing TLR ligands. Engagement of all the TLR receptors studied triggered *Jmjd3* expression. The highest induction was caused by the TLR4 agonist, LPS, when cells were treated with the optimal conditions. This can be

explained by the fact that TLR4 is the only TLR that can signal both through the MyD88 dependent and independent pathways (Kagan et al., 2008), and therefore can activate NF-κβ and finally *Jmjd3* expression by two different routes. Moreover, LPS has been described to be the most potent stimulant of microglia, compared to TLR2 and TLR9 agonists (Ebert et al., 2005). However, the TLR2/1 and TLR2/6 heterodimers agonists FSL-1 and Pam3CSK4 also induced a considerable up-regulation of *Jmjd3*.

That higher *Jmjd3* mRNA levels were achieved through TLR4 ligation, followed by TLR2 stimulation, is relevant for both EAE and MS. In EAE, TLR2 and TLR4 mediated mechanisms have been implicated in the initiation of disease. CFA, the adjuvant used to induce EAE, contains mycobacterium tuberculosis extracts that activate the innate immune response through the TLR2 and TLR4 receptors (Stenger and Modlin, 2002). Additionally, pertussis toxin functions as a ligand for TLR4 (Kerfoot et al., 2004). Although no direct association between TLR endogenous ligands and MS has been described so far, the presence of fibrinogen (a TLR4 endogenous ligand) and HMGB1 (a TLR2 and TLR4 self-stimulant) has been documented in MS and EAE active lesions (Andersson et al., 2008; Gay and Esiri, 1991). Gangliosides (neuronal membrane components released after neuronal damage) and oxidised by-products of myelin breakdown by ROS, which are present in the CSF and serum of MS patients, have been described to bind to TLR4 and TLR2 receptors, respectively, and activate microglia (Diestel et al., 2003; Farez et al., 2010; Jou et al., 2006). Furthermore, whilst healthy white matter does not contain TLRs, active MS lesions show a high expression of TLR4 on microglia (Bsibsi et al., 2002). In addition, it is hypothesised that systemic circulation of bacterial endotoxins after urinary tract infections might cause microglial and CNS macrophage activation through TLR binding (Wolfson and Talbot, 2002). However, despite stimulation of other TLRs was also found to up-regulate *Jmjd3*, and TLR2 ligation also seems to be important for the immunopathogenesis of MS and EAE, only LPS was used to further

characterise the role of Jmjd3 expression in microglial activation. This was decided because LPS was the most potent inducer of the enzyme, and could therefore facilitate the detection of any possible effects of Jmjd3 up-regulation in functional experiments.

Nevertheless, before proceeding with functional experiments, the effect on *Jmjd3* expression of other more physiologically relevant stimuli than LPS, such as pro-inflammatory cytokines and chemokines previously described to be relevant for MS and EAE pathology, was also studied. IL-1β and IL-8 are both NF-κB inducers (Allan et al., 2005; Manna and Ramesh, 2005) and therefore significantly increased *Jmjd3* expression, although this was to a lesser extent than the TLR agonists. Depending on the nature and intensity of the stimulus, microglia can show different activated effector phenotypes, which range from neurotoxic to protective. During autoimmune and infectious CNS inflammation, TLR stimulation of microglial cells leads to strong innate immune defense reactions that release neurotoxic factors and induce a higher expression of *Jmjd3*, which could potentially help to potentiate the pro-inflammatory activation. Conversely, minor insults that are accompanied by a moderate production of one or few cytokines/chemokines (like neuronal cell death during development or the damage of a few synapses) bring about a protective activated phenotype with more contained responses and a lower induction of *Jmjd3* that promotes tissue repair and survival of neurons and glia.

Even though the IFNγ signalling pathway does not involve the activation of NF-κB directly, the addition of this cytokine alone also induced a modest increase of *Jmjd3* expression. This can be explained by an autocrine process involving the IFNγ-induced rapid synthesis of the NF-κB signalling cytokines IL-1β and TNFα, which have been reported to contribute to the priming effect of IFNγ on LPS-NF-κB activation (Held et al., 1999). Both TNFα and IL-1β are secreted by activated microglia (Block et al., 2007) and both modestly up-regulate *Jmjd3*, as described by De Santa *et al*. in the case of TNFα (De Santa et al., 2007) and as demonstrated in figure 3.6 for IL-1β. Furthermore, the IFNγ induced protein kinase dsRNA-regulated (PKR) signals via

NF-κB, which could constitute another mechanism by which IFNγ up-regulates *Jmjd3* through an intermediary molecule (Meurs et al., 1990; Zamanian-Daryoush et al., 2000).

IFNγ has also been shown to be a priming agent in monocytes and macrophages that sensitises them for an increased response to successive stimuli (Hayes et al., 1995; Pace et al., 1983). Priming by IFNγ in human monocytes has been described to start from 2 hours pre-treatment with IFNγ before LPS addition. However, it was also shown that there was more *TNFα* expression with the simultaneous addition of LPS and IFNγ for 2 hours than with LPS alone (Hayes et al., 1995). This "early" priming can be explained by the fact that around 2 hours after incubation, IFNγ starts increasing LPS induced transcription through an enhancement of NF-κB activity, secondary to an augment of its DNA binding capacity and a more rapid IκB-α (NF-κB inhibitor) degradation, both of which persists for around 30 minutes (Hayes et al., 1995; Held et al., 1999). Therefore, the higher *Jmjd3* expression observed after administration of LPS plus IFNγ rather than LPS alone, after 2 and 4 hours incubations, suggests an early priming role of IFNy in microglial cells, just as it has been documented for macrophages (Pace et al., 1983). Since the 8 and 24 hours incubation time points between LPS and LPS plus IFNγ did not show any difference, it could be hypothesised that priming does not increase the *Jmjd3* mRNA stability, which is another mechanism by which priming can operate (Hayes et al., 1995).

Regardless of the strong increase in Jmjd3 expression triggered by LPS plus IFNγ in microglia *in vitro*, there were no global variations on H3K27me3. This led to the assumption that Jmjd3's effects were carried out at a local gene level in a limited number of targets. A group of inducible inflammatory and cytotoxic genes that were demonstrated to be up-regulated in LPS plus IFNγ activated microglia, some of which had been reported to be dependent on Jmjd3 for their expression in macrophages (De Santa et al., 2009), were selected as possible Jmjd3 target candidates. These included *Ccl3*, *Ccl5*, *Cxcl11*, *Il6*, *Il12b* and *Nos2*, which were

additionally chosen on the basis of their importance for the immunopathogenesis of MS and EAE. IL-6 is a pro-inflammatory cytokine secreted by activated macrophages and microglia that has been detected at elevated levels in the serum of MS patients (Özenci et al., 2000). This cytokine, along with TGF-β, induces naïve CD4 T cells to differentiate into T_H17 cells (Bettelli et al., 2006), which are the main effector T cells in the EAE and MS autoimmune reaction (Komiyama et al., 2006; Tzartos et al., 2008). IL-12 is also a pro-inflammatory cytokine that is elevated in the CSF and brain lesions of MS patients and mediates T_H1 polarisation of CD4 T lymphocytes (the other T cell subset that orchestrates inflammation in the early stage of MS) (Lock et al., 2002; Segal et al., 1998). CCL3 (or macrophage inflammatory protein 1 alpha, MIP-1α) and CCL5 (or Rantes) are chemokines involved in the CNS recruitment and activation of leukocytes and T lymphocytes. They are also both elevated in the CSF as well as in active lesions in MS, and in EAE are associated with disease progression (Boven et al., 2000; xF et al., 1999). NOS2, or inducible nitric oxide synthase (iNOS), is an enzyme that generates large amounts of NO, a bioactive free radical that mediates microglia-induced cytotoxicity in MS and EAE (Chao et al., 1992; Merrill et al., 1993).

De Santa el al. reported that the Jmjd3 mediated regulation of gene expression of *Ccl3, Ccl4, Ccl5, Ccl9, Cxcl11, Il12b,* and *Il6* in pro-inflammatory activated macrophages was independent of the enzyme's demethylase activity (De Santa et al., 2009). However, Ishii *et al*. demonstrated that in alternative activated macrophages, Jmjd3 binds to the promoter of M2 marker genes and increases their expression by demethylating H3K27me3 (Ishii et al., 2009). It was not known which mechanism occurred in microglia, or even if Jmjd3 or any other H3K27me3 demethylase was involved in the activation process of these cells. To answer this, the putative recruitment of the newly synthesised Jmjd3 to the promoter of the selected candidate genes and its potential effect on H3K27me3 levels were investigated through ChIP qPCR assays in LPS plus IFNγ treated microglia. Jmjd3 was enriched in the TSS of *Il6*, *Ccl3*,

Ccl5, *Nos2* and *Gbp6* (positive control), roughly in ascending order. The *Il12b* region analysed did not show a significant increase in Jmjd3 co-localisation in activated microglia, which could either mean that the gene is not a Jmjd3 target in microglia or that in this cell type Jmjd3 binds to a different region. Importantly De Santa *et al.* also reported that Il12b was only modestly enriched with Jmjd3 when compared to highly enriched genes such as *Gbp6* and *Nos2* (De Santa et al., 2009), which were also the highest enriched genes in this study. Therefore it could be that the sensitivity of the anti-Jmjd3 ChIP-qPCR assay used was not high enough to detect the relatively low *Il12b* binding. Interestingly, no Jmjd3 binding to *Ccl3* had been described before in macrophages, but the enzyme has been shown to be necessary for the expression of Ccl3 in T cells (Miller et al., 2010). These differences in Jmjd3 co-localisation may be explained by the fact that, although related, microglia and macrophages are different cell types and therefore could show different epigenetic regulation of activation.

Given that all the genes that were enriched with Jmjd3 (*Il6, Ccl3, Ccl5* and *Nos2)* also showed a decrease in H3K27me3, it could be proposed that the probable mechanism of action of Jmjd3 in the regulation of the expression of such genes might be related to its demethylase enzymatic activity. However *Cxcl11* and *Il12b* also showed a reduction in H3K27me3 despite not co-localizing with Jmjd3. Therefore, it cannot be stated that the reduction in H3K27 methylation at the analysed genes was caused by the enzymatic activity of Jmjd3 as it could also be explained by a reduction in H3 caused by nucleosome lost secondary to gene activation.

In addition to being a H3K27me3 demethylase, Jmjd3 is also involved in the regulation of the recruitment of Pol II and the transcriptional elongation of target genes (Chen et al., 2012; De Santa et al., 2009), as well as having a chromatin remodelling function (Miller et al., 2010). Hence, whether or not the potential Jmjd3 involvement in the regulation of transcription in activated microglia is dependent on its histone modifying activity, or on one of its other

described functions, requires further investigation. ChIP-sequencing analysis could shed some light in this respect. However, before proceeding with this method, the up-regulation of Jmjd3 in activated microglia *in vivo* in a mouse model of MS was investigated along with its potential functional consequences, as described in the following chapters.

4. Results

4.1. Introduction

In the acute phase of MS, microglia play a central role in both the adaptive autoimmune response, by secreting cytokines that promote T_H1 and T_H17 differentiation of T cells, and in the unspecific innate inflammatory response that damages oligodendrocytes and neurons through the release of compounds such as NO and ROS. Over time the acute inflammatory response gives way to diffuse, chronic inflammation, characterised by widespread, persistent activation of parenchymal microglia. In MS and CR-EAE, this chronic activation of microglia is associated with damage to the brain cortex and NAWM, which in turn cause progression of disease and permanent neurological disability (Kutzelnigg et al., 2005; Rasmussen et al., 2007). The mechanisms behind persistent microglial activation have not been characterised.

Classical EAE is a T cell-driven disease, in which antigen-specific, MHC class II restricted T cells induce CNS inflammation and tissue injury through the activation of microglia and macrophages (Mendel et al., 1995; Wekerle, 1993). Although mouse models cannot recapitulate MS pathogenesis entirely, EAE allows research into the molecular and inflammatory mechanisms behind the disease. Depending on the mouse strain and on the immunising antigen, different types of EAE can be induced, which mirror different clinical and histopathological aspects of MS. C57BL/6 mice develop chronic monophasic EAE after immunisation with the myelin peptide $MOG_{35–55}$. This typically starts around 10 days post immunisation and debuts with weakening or paralysis of the tail that progresses to hind limb paralysis at the peak of disease (usually 14-18 days after immunisation) (Mendel et al., 1995).

In contrast, Biozzi ABH mice immunised with MOG or PLP epitopes emulsified in adjuvant develop CR-EAE, which has important similarities to MS with a relapsing-remitting disease course followed by a secondary progressive phase. In Biozzi ABH mice the onset of clinical

signs occurs around 11 to 16 days post-immunisation and active disease is also characterised by paralysis of the hind limbs and tail. Around 80% of the mice develop a relapsing-remitting disease course that remits almost completely after the disease onset, but some residual tail atony can be observed. This residual neurological deficit increases after subsequent relapses. As it has been demonstrated in other murine MS models, microglial activation also precedes T-cell infiltration. Axonal loss is evident from the acute phase of disease, independently of demyelination. After each relapse, demyelination and axonal loss increases, correlating with rising residual motor deficits in remissions. Following a series of relapses, mice enter a chronic progressive phase with permanent neurological disability and relative absence of inflammation, as is observed in progressive human MS (Amor et al., 1994; Baker et al., 1990; Jackson et al., 2009) .

Having demonstrated that Jmjd3 is up-regulated in microglia activated by pro-inflammatory stimuli *in vitro* and that it is recruited to the promoters of immune response and inflammation related genes, it was subsequently examined if this Jmjd3 induction could also been observed *in vivo* in the experimental model of MS, EAE. Since one of the aims of this thesis was to identify putative epigenetic mechanisms contributing to chronic microglial activation during MS remissions and the progressive phase of the disease, induction of CR-EAE in Biozzi ABH was therefore used to study such processes.

4.2. Results

4.2.1. Jmjd3 is up-regulated in activated microglia from mice with acute EAE

Following the observation that primary murine microglia up-regulate Jmjd3 after incubation with pro-inflammatory substances *in vitro*, it was investigated whether activated microglia also up-regulate Jmjd3 in EAE.

The yield of mononuclear cells obtained from the CNS of adult mice is low, even after induction of EAE. Therefore, in order to measure the protein level of Jmjd3 in microglia and CNS infiltrating macrophages separately, a method that does not require high number of cells and that allows the differentiation of the two populations within the same sample was needed. There are no specific microglial markers that can be used to distinguish the two cell populations by immunohistochemistry. However, several groups have successfully employed a method to differentiate resting microglia from macrophages in CNS mononuclear cells samples based on measuring the expression of the surface markers CD11b and CD45 by flow cytometry. Consequently, the suitability for flow cytometry analysis of the polyclonal anti-Jmjd3 antibody used for ChIP and immunocytochemistry in the previous chapter was initially tested in HEK 293T cells transiently transfected with a pCMV-HA-*Jmjd3* plasmid. The transfected cells were stained intracellularly with the anti-Jmjd3 polyclonal antibody, followed by secondary fluorescent antibody staining and flow cytometry analysis. To test for transfection efficiency and specificity of the anti-Jmjd3 antibody binding, an anti-HA monoclonal antibody was also used.

68.9% of the transfected cells were positive for HA tag expression and 61.7% (42.4% of the total cells) of these HA positive cells were also positive for Jmjd3 (Figure 4.1). The anti-Jmjd3 antibody was able to detect the higher expressing cells, whereas the lower expressing cells could only be detected by the anti-HA antibody. Therefore, whilst the anti-HA antibody was more sensitive than the anti-Jmjd3 antibody, the anti-Jmjd3 antibody was shown to be specific. Basal Jmjd3 protein expression was also detected in non-transfected cells (as previously observed by immunohistochemistry in non-stimulated primary microglia in figure 3.8). However, the Jmjd3 antibody was able to specifically recognise the recombinantly expressed protein above this background.

Two other polyclonal anti-Jmjd3 commercial antibodies and an antibody kindly provided by Dr. Kristian Helin (BRIC, Copenhagen) were also tested for flow cytometry. These generated inferior detection values and therefore it was decided that the anti-Jmjd3 antibody previously used in the study for ChIP and immunocytochemistry was the most suitable for Jmjd3 detection by flow cytometry.

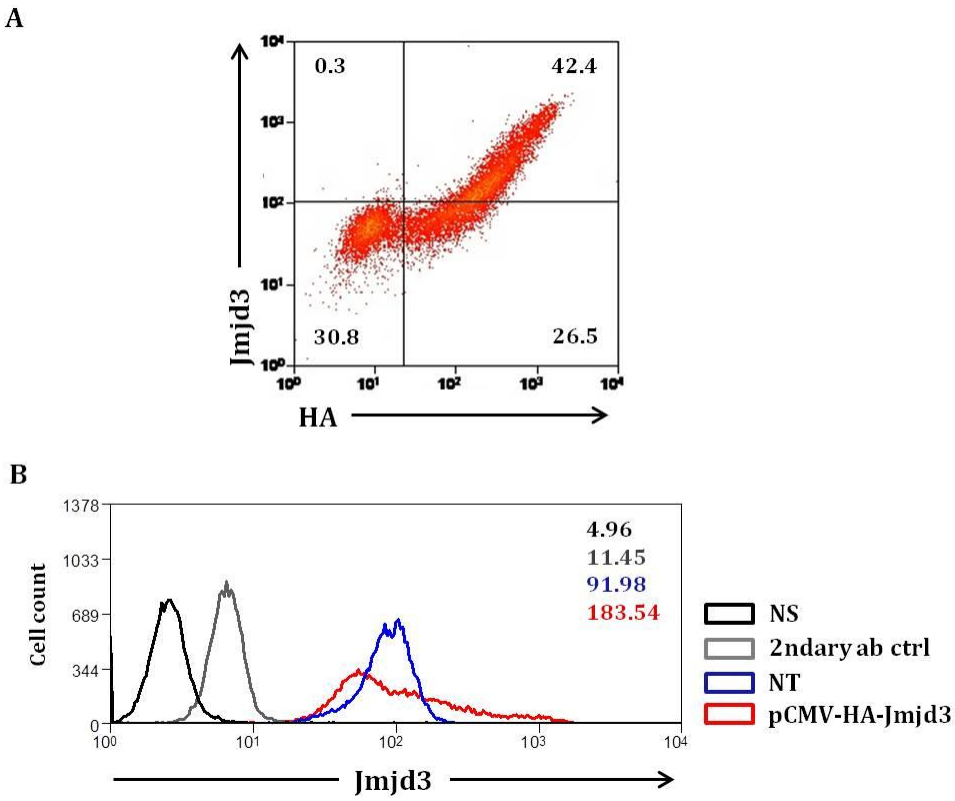


Figure 4.1. Jmjd3 flow cytometry optimisation. HEK 293T cells were transfected with a pCMV-HA-Jmjd3 plasmid. After 48 hours, the cells were intracellularly immunostained with an anti-Jmjd3 polyclonal antibody and an anti-HA monoclonal antibody, followed by flow cytometry analysis. (A) Representative Jmjd3 and HA tag dot plot of transfected cells. (B) Overlay of representative Jmjd3 fluorescence histograms. The mean fluorescence intensity (MFI) for each sample is shown.

Having tested the sensitivity and specificity of the Jmjd3 antibody, it was then initially used to analyse the Jmjd3 protein expression in microglia isolated from a small group (n= 5) of MOG_{35-}

55 immunised EAE C57BL/6 mice at peak of disease in comparison to the control group (CFA alone plus PBS) (n= 4) and naïve (n= 3) (healthy, non immunised) mice.

The brain and spinal cords were dissected and the CNS mononuclear cells (which include microglia and infiltrating leukocytes) were isolated by percoll gradient following the protocol previously demonstrated by Cardona *et al.* (Cardona et al., 2006a) and described in section 2.6.5. Sedgwick *et al*. described a flow cytometry method to discriminate resting microglia from macrophages based on differences in the level of CD45 expression of $CD11b^+$ cells, which has been replicated in several other reports. Ramified, non activated microglia show a relatively low CD45 expression and have been characterised as $CD11b^+$ $CD45^{low}$, while other CNS and peripheral macrophages display a $CD11b^+$ $CD45^{high}$ phenotype (Becher and Antel, 1996; Sedgwick et al., 1991; Stevens et al., 2002). However, it has also been shown that microglia up-regulate CD45 following activation under inflammatory conditions (Ponomarev et al., 2005; Sedgwick et al., 1991). Therefore, to be able to differentiate resting or surveying microglia from activated microglia and macrophages, the isolated CNS mononuclear cells were stained with anti-CD11b and anti-CD45.2 monoclonal antibodies. To then determine the percentage of cells which had become activated following immunisation, the CNS mononuclear cells were first gated based on their expression of CD11b. $CD11b^+$ cells, all of which were $CD45^+$, were further subdivided by their CD45 expression, with $CD11b^+$ $CD45^{low}$ cells constituting resting microglia and $CD11b^+$ $CD45^{high}$ cells comprising the activated microglia/macrophages population (figure 4.2A).

In the naïve mice CNS samples, 9.3% of the $CD11b^+$ cells were $CD45^{high}$ (activated microglia/macrophages). In the CFA alone plus PBS control CNS samples, the number of $CD11b^+$ $CD45^{high}$ cells was slightly higher than in the naïve mice (17.7%). As CFA contains TLR ligands that can induce microglial activation in the absence of antigen (Okun et al., 2009), this is therefore likely to be the cause of the observed increase of activated cells. As expected, the

MOG_{35-55} EAE C57BL/6 mice at peak of disease had the greatest population of $CD11b^{+}$ $CD45^{high}$ cells than both the control and naïve mice (51.6%). This increase reflects the activation of a proportion of the resting microglial cells ($CD11b^{+}$ $CD45^{low}$) and macrophage infiltration secondary to the CNS auto-inflammatory process (Figure 4.2B).

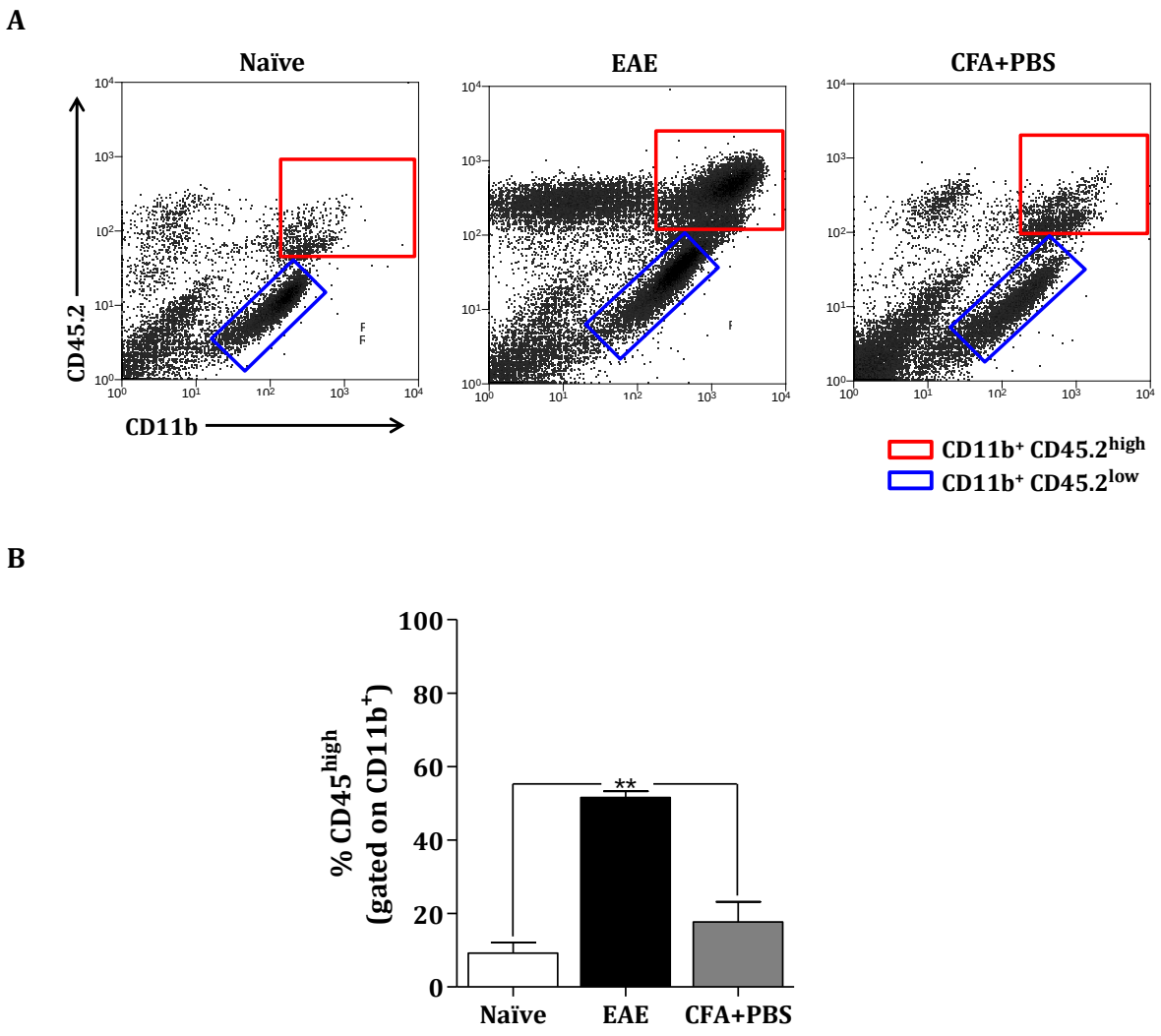


Figure 4.2. Resting microglia and activated microglia/macrophage populations in naïve, control and EAE mice. The brain and spinal cord mononuclear cells from naïve, control (CFA+PBS) and MOG_{35-55} EAE (at peak of disease) C57BL/6 mice were isolated, immunostained for the cell surface markers CD45.2 and CD11b, and analysed by flow cytometry. The $CD11b^+$ cells were gated based on their level of CD45 expression into $CD11b^+$ $CD45^{low}$ (resting microglia) and $CD11b^+$ $CD45^{high}$ (activated microglia/macrophages) populations. (A) Representative CD11b and CD45.2 dot plots are shown. (B) Graph showing the percentage of activated microglia/macrophages ($CD45^{high}$ cells) in $CD11b^+$ cells from naïve (n= 3), MOG_{35-55} EAE (n= 5) and CFA+PBS (n= 4) C57BL/6 mice from CNS samples. Bars represent mean ± standard error. **$p < 0.01$.

As has been previously described, the $CD11b^+$ $CD45^{low}$ resting microglial cells were found to be small and homogenous, as demonstrated by their forward and side scatter, while the

CD11b^{+} CD45^{high} population displayed a characteristic activated macrophage like-phenotype with a large size and increased granularity (Figure 4.3) (Ford et al., 1995; Ponomarev et al., 2005).

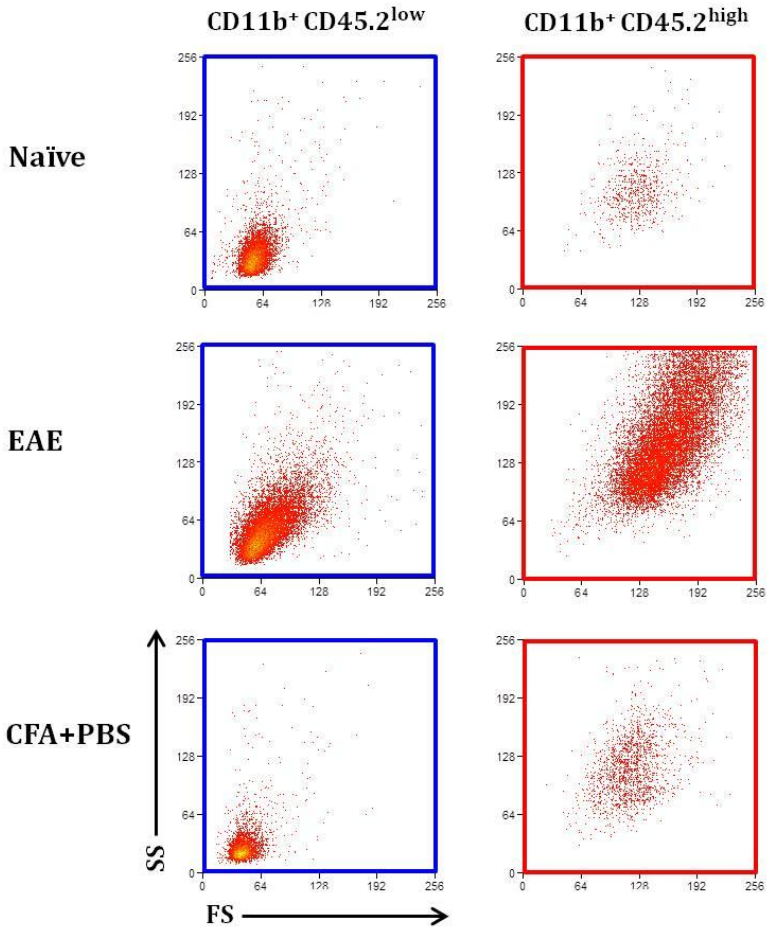


Figure 4.3. Activated microglia/macrophages have a large size and increased granularity. The brain and spinal cord *mononuclear cells* from naïve, control (CFA+PBS) and MOG_{35-55} EAE (at peak of disease) C57BL/6 mice were isolated, immunostained for the cell surface markers CD45.2 and CD11b, and analysed by flow cytometry. The cells were gated based on their level of CD45 expression into CD11b^{+} CD45^{low} (resting microglia) and CD11b^{+} CD45^{high} (activated microglia/macrophages) populations. Representative forward and side scatter dot plots are shown.

To then be able to measure the Jmjd3 expression in the CD11b^{+} CD45^{low} (resting microglia) and CD11b^{+} CD45^{high} (activated microglia/macrophages) populations, the surface staining was

followed by permeabilisation and intracellular staining with the anti-Jmjd3 antibody and a secondary fluorescent antibody, after which cells were analysed by flow cytometry. While the few $CD11b^{+}$ $CD45^{high}$ cells isolated from the CNS of naïve C57BL/6 mice did not seem to have any changes in the expression of Jmjd3, the activated microglia and macrophages from control CFA alone immunised mice showed a small up-regulation of Jmjd3 compared to the $CD11b^{+}$ $CD45^{low}$ population (MFI fold change of 1.17). However, this was not significant. On the other hand, the activated microglia and infiltrating macrophages from MOG_{35-55} EAE C57BL/6 mice exhibited an increase in Jmjd3 expression compared to the resting microglia population (MFI fold change of 1.7) that was significantly higher than the MFI fold change observed in both the control ($p= 0.0003$) and naïve ($p=0.0005$) mice (Figure 4.4 A and B). The secondary fluorescent antibody showed some un-specific staining, however this was lower than the positive staining observed with the anti-Jmjd3 antibody (Figure 4.4 A).

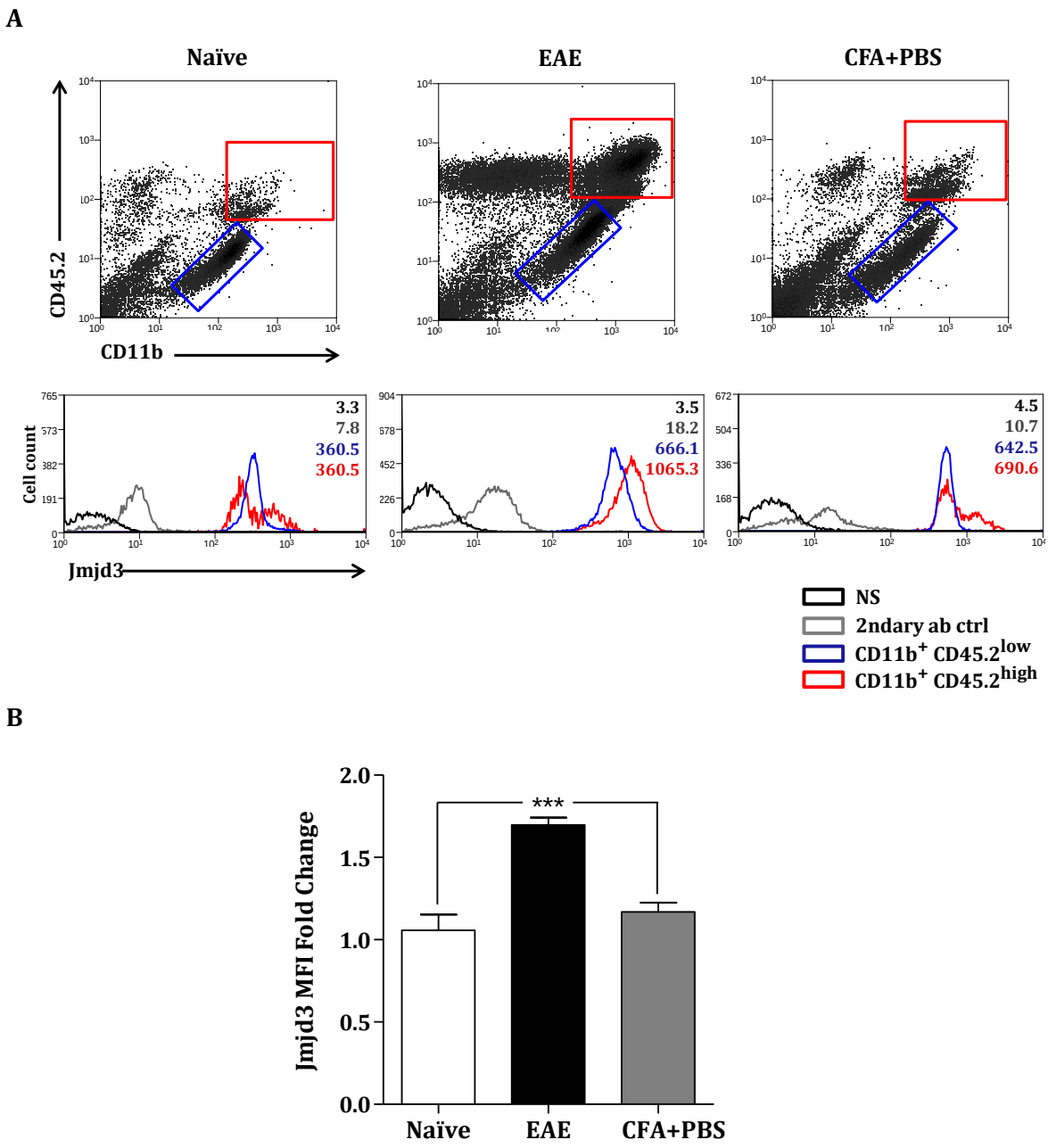


Figure 4.4. Jmjd3 is up-regulated in activated microglia/macrophages from EAE mice. Flow cytometric analysis of Jmjd3 expression in CD11b+ CD45$^{low/high}$ CNS-derived mononuclear cells isolated from naïve, control (CFA+PBS) and MOG$_{35-55}$ EAE (at peak of disease) C57BL/6 mice. (A) Representative CD45.2/CD11b dot plots with gates on CD11b^{+} CD45^{low} (resting microglia) and CD11b^{+} CD45^{high} (activated microglia/macrophages) populations are shown at the top. Representative overlays of Jmjd3 fluorescence histograms with the mean fluorescence intensity values of non-stained (NS) cells, secondary antibody only stained cells, resting microglia and activated microglia/macrophages are shown on the bottom. (B) Graph showing the fold change in mean fluorescence intensity (MFI) of Jmjd3, which was calculated by dividing the activated microglia/macrophages population (CD11b^{+} CD45^{high}) MFI by the resting microglia population (CD11b^{+} CD45^{low}) MFI of each mouse. Naïve n=3. EAE n=5. CFA+PBS n=4. Bars represent mean ± standard error. ***$p < 0.001$.

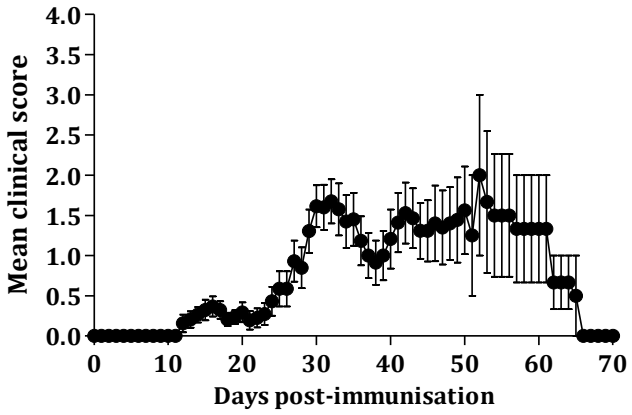
4.2.2. Analysis of Jmjd3 expression in activated microglia from CR-EAE mice

To then explore if Jmjd3 up-regulation could be involved in the chronic activation of microglia a CR-EAE mouse model was used to compare the expression of Jmjd3 in activated microglia/macrophages between early (disease onset and first relapse) and late disease stages (second remission). Previous EAE studies with Biozzi mice performed in our group have shown that the incidence of CR-EAE in this model is approximately 80% (Vergo et al., 2011). Since the yield of mononuclear cells isolated from the CNS of adult mice is low (even after induction of EAE), 3-4 mice in the same disease stage were pooled together per sample in order to obtain enough cells for following experiments. Therefore, to have three samples per disease stage, 40 Biozzi ABH mice were immunised with MOG_{35-55} emulsified in CFA, as described in the general materials and methods section 2.6.4. As expected, the immunised Biozzi ABH mice showed a relapsing-remitting disease course, with onset of clinical signs occurring from day 12 post-immunisation. Mice were sacrificed at different time points throughout the disease course, including disease onset (between day 20 to 27 post-immunisation), first relapse (between day 30 to 42 post-immunisation) and second remission (between day 45 to 64 post-immunisation) (Figure 4.5 A and B). Naïve and CFA plus PBS immunised Biozzi ABH mice were also taken as controls at day 40 post-immunisation.

Following the initial peak of disease after immunisation, a remission phase was defined as improvement from the preceding relapse (or initial disease episode) with a sustained (3 or more days) decrease in clinical score of at least 1.0. Relapses were identified by sustained (more than 3 days) increase of more than 1.0 clinical score from the baseline score of the previous remission. The baseline clinical status between each remission and the next relapse was assessed using the same scale. Mice were also weighed daily. Changes in body weight were used as an indication of an imminent relapse or recovery. Mice usually start losing 1 to 2 g of their body weight a couple of days before the onset of EAE and before each relapse. The

weight loss continues with the increase in clinical score and can reach up to 20% of the pre-EAE onset body weight at the peak of disease. After the peak of disease is reached, mice slowly start gaining weight again, often before the clinical score improves (Jackson et al., 2009) (Figure 4.5 B).

A



B

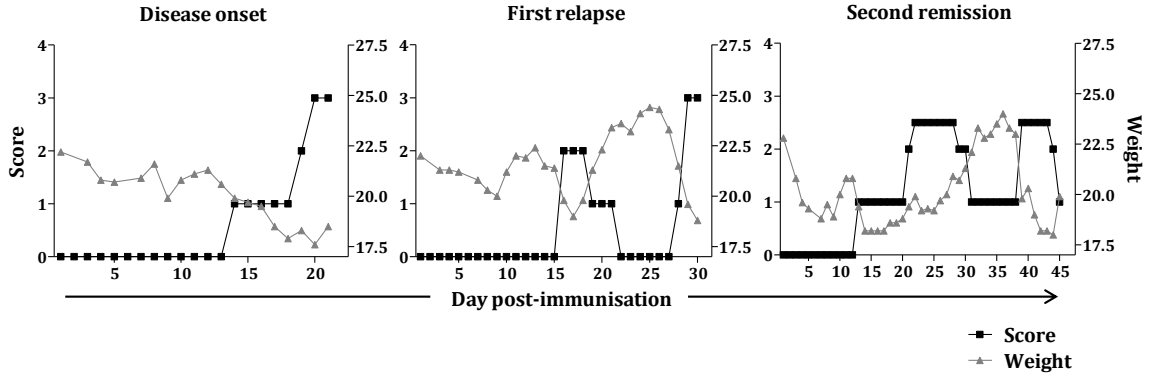


Figure 4.5. Biozzi ABH mice immunised with MOG_{35-55} develop CR-EAE. (A) Clinical scores (means ± s.e.m.) for CR-EAE Biozzi mice from day 0 to 70 after immunisation with MOG_{35-55} (n = 40) (B) Representative graphs of the clinical course and correlation with body weight of CR-EAE Biozzi ABH mice taken at the different time points throughout the disease course.

The brains and spinal cords of the sacrificed mice were dissected and the CNS mononuclear cells were isolated. Cell subsets were then sorted by flow cytometry into $CD11b^{+}$ $CD45^{low}$

(resting microglia), $CD11b^{+}$ $CD45^{high}$ (activated microglia/macrophages) and $CD11b^{-}$ $CD45^{+}$ (other CNS infiltrating hematopoietic cells including T lymphocytes) with the help of Craig Waugh (WIMM flow cytometry core facility).

In the naïve mice, only a small percentage of $CD11b^{+}$ cells were $CD45^{high}$, activated microglia/macrophages (9.6%). There was an increase in the percentage of activated cells in the CFA control mice (36%), likely secondary to CFA-mediated TLR stimulation (as it was also observed in the preceding C57BL/6 EAE study). As a result of microglial activation and macrophage infiltration caused by disease activity, this percentage of activated $CD11b^{+}$ $CD45^{high}$ cells increased further in the samples from disease onset (40.3%) and first relapse (48%) of MOG_{35-55} CR-EAE mice, in comparison to control mice. Upon second remission, the proportion of activated microglia/macrophages showed a reduction (24.8%), compared to samples taken in episodes of active disease. However, this activated population remained significantly higher than in naïve samples ($p= 0.013$) (Figure 4.6). This indicates that, as previously described in the literature, a proportion of microglia remain activated even after the clinical score improves (Rasmussen et al., 2007).

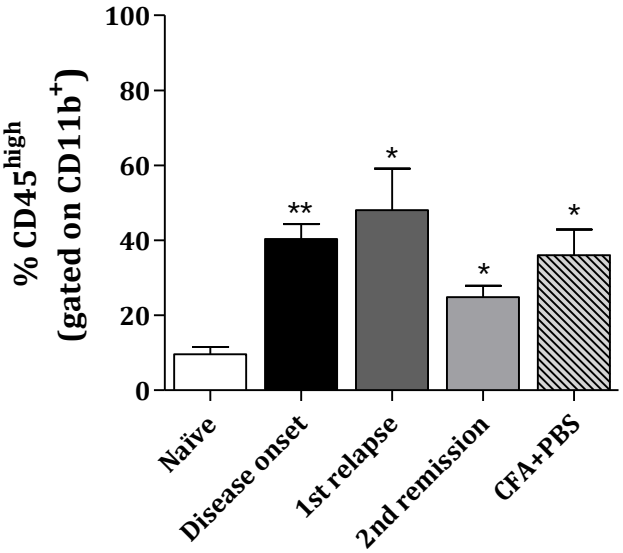


Figure 4.6. Percentage of activated microglia/macrophages in CD11b$^+$ cells from different disease stages of CR-EAE. MOG_{35-55} CR-EAE Biozzi mice were sacrificed at three different time points in the disease course (3 to 4 mice were pooled together per sample), including disease onset, first relapse and second remission. Naïve and mice immunised with CFA alone plus PBS were also included in the study as controls. The CNS mononuclear cells were isolated, immunostained for the surface markers CD45.2 and CD11b and sorted by flow cytometry into CD11b$^+$ CD45^{low} (resting microglia), CD11b$^+$ CD45^{high} (activated microglia/macrophages) and CD11b$^-$ CD45$^+$ populations. The graph shows the percentage of CD45^{high} cells in the CD11b$^+$ population of the samples from the different disease stages. n=3 samples/group. Bars represent mean ± standard error. *$p < 0.05$. **$p < 0.01$. (compared to naïve).

4.2.2.1. *Jmjd3* is up-regulated at the mRNA level in activated microglia from CR-EAE mice

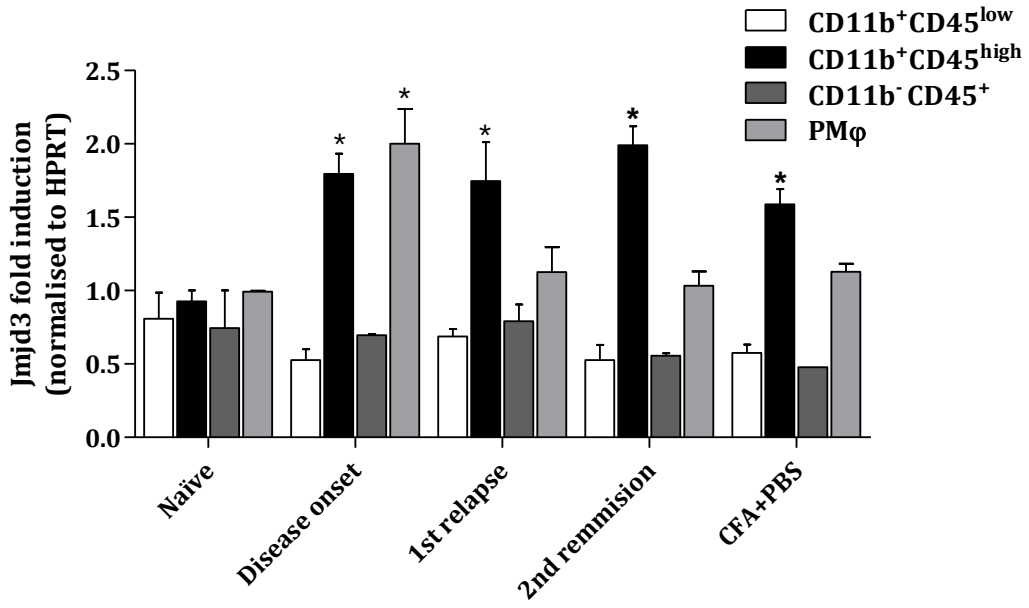
The *Jmjd3* expression in the three CNS mononuclear cells subsets isolated per sample was then determined by qRT-PCR. Peritoneal macrophage (PMφ) samples from the different disease stages were also harvested and analysed for *Jmjd3* expression.

The *Jmjd3* mRNA levels in the resting microglia (CD11b$^+$ CD45^{low}) and the CD11b$^-$ CD45$^+$ cells (other CNS infiltrating hematopoietic cells, including T lymphocytes) were low and comparable across samples from the different disease stages. In contrast, in the activated

microglia/macrophages population ($CD11b^+$ $CD45^{high}$) the *Jmjd3* expression was significantly up-regulated in all of the disease course time points analysed, compared to the naïve mice samples (disease onset fold induction= 1.8, first relapse fold induction= 1.4 and second remission fold induction= 2). There were no significant differences in the level of expression between disease stages. The *Jmjd3* mRNA levels were also up-regulated in activated microglia/macrophages from control mice immunised with CFA alone plus PBS (1.6 fold induction above naïve). This indicates that *Jmjd3* induction in this population was not exclusively disease dependant, but rather a general microglial activation event after exposure to pro-inflammatory stimuli. Furthermore, the peritoneal macrophage samples from disease onset showed a significant up-regulation of *Jmjd3* (2 fold induction above naïve), most probably also secondary to CFA mediated activation. Importantly, activated microglia/macrophages from mice in second remission (which were taken approximately at the same day post-immunisation as mice immunised with CFA alone plus PBS) showed modestly higher *Jmjd3* expression compared to CFA alone controls (2 vs. 1.6 fold induction above naïve). Even though this difference was not significant ($p=0.0921$), it suggested that this further *Jmjd3* up-regulation was disease specific (Figure 4.7A).

The expression of *Utx* in all the samples was also analysed by qRT-PCR as a negative control. As in the *in vitro* studies with primary murine microglia, no up-regulation of *Utx* was observed in the activated microglia/macrophages population in any of the disease time points studied. The *Utx* mRNA levels also stayed constant and low in the $CD11b^+$ $CD45^{low}$, $CD11b^-$ $CD45^+$ and peritoneal macrophage populations throughout the disease course (Figure 4.7B).

A



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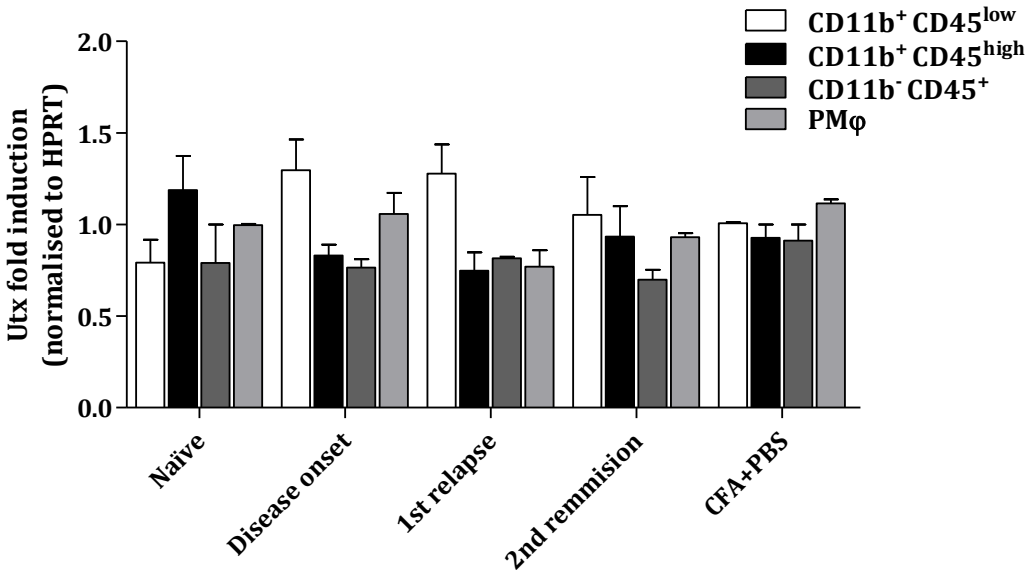

Figure 4.7. *Jmjd3* is up-regulated in activated microglia/macrophages from CR-EAE mice independently of the disease stage. MOG_{35-55} CR-EAE Biozzi mice were sacrificed at disease onset, 1st relapse and 2nd remission (3-4 mice were pooled per sample). Control CFA alone plus PBS mice were sacrificed on day 40. The CNS mononuclear cells were isolated and immunostained for the surface markers CD45.2 and CD11b. The $CD11b^{+}CD45^{low}$ (resting microglia), $CD11b^{+}CD45^{high}$ (activated microglia/macrophages) and $CD11b^{-}$ $CD45^{+}$ (mainly T cells) populations were sorted by flow cytometry and used for RNA isolation. Peritoneal macrophages (PMφ) were also harvested from each group. The mRNA levels were measured by qRT-PCR, normalised to HPRT and the (A) *Jmjd3* and (B) *Utx* (as control) fold induction was calculated compared to the naïve mice samples of each cell population. n=3 samples/group. Bars represent mean ± standard error. *$p < 0.05$

4.2.2.2. Jmjd3 is up-regulated at the protein level in activated microglia from CR-EAE mice

To establish if the expression of Jmjd3 at the protein level is also increased in activated microglia/macrophages from CR-EAE Biozzi ABH mice independently of the disease stage, isolated CNS mononuclear cells were stained for the surface markers CD11b and CD45.2, followed by intracellular staining for Jmjd3. The $CD11b^{+}$ CNS mononuclear cells were gated based on their level of CD45 expression into $CD11b^{+}$ $CD45^{low}$ (resting microglia) and $CD11b^{+}$ $CD45^{high}$ (activated microglia/macrophages) populations and the mean fluorescence intensity of the Jmjd3 staining was determined for each subset.

In accordance with the qRT-PCR data, in immunised mice, the Jmjd3 expression at the protein level was higher in activated microglia/macrophages compared to the resting microglial population (Figure 4.8A). However, the increase in Jmjd3 was independent of the disease stage, as it was observed across all samples from different time points throughout the disease course. Whilst activated microglia/macrophages from immunised mice showed significantly more Jmjd3 protein expression than naïve mice, this up-regulation was not significantly higher when compared to the CFA alone control group (as was observed in the mRNA data). Once more, these data suggest that the increase in Jmjd3 is independent of disease, at least partially, because the Jmjd3 expression in the second remission samples (Jmjd3 MFI= 1.5) was modestly higher than in the CFA controls (Jmjd3 MFI= 1.35) (also as previously seen in the mRNA data). However, the difference was not significant (p= 0. 1693) (Figure 4.8B).

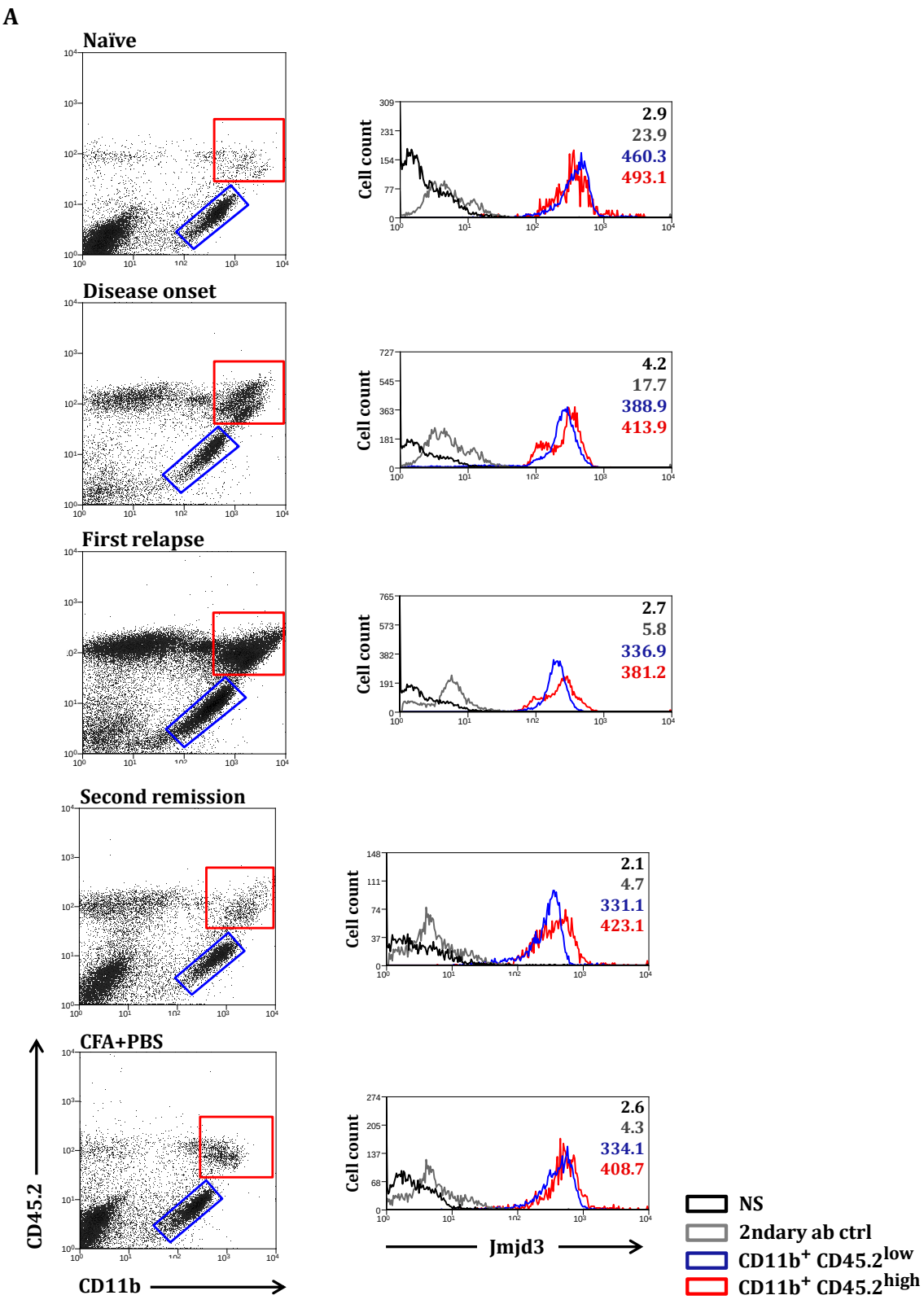

Figure 4.8. Jmjd3 is up-regulated in activated microglia/macrophages from CR-EAE mice independently of the disease stage.

B

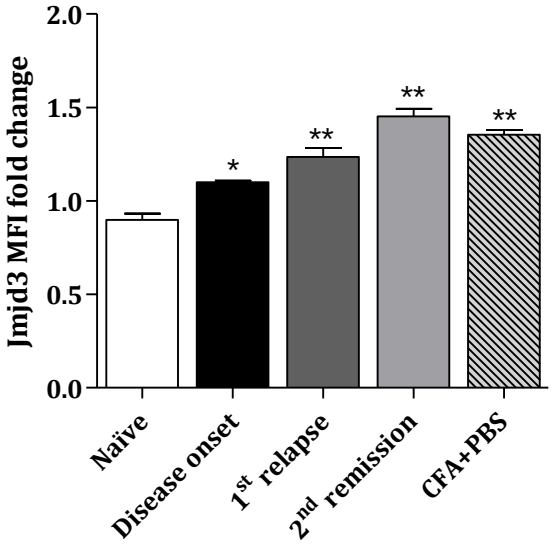

Figure 4.8 continued. Jmjd3 is up-regulated in activated microglia/macrophages from CR-EAE mice independently of the disease stage. MOG_{35-55} CR-EAE Biozzi mice were sacrificed at disease onset, 1st relapse and 2nd remission (3-4 mice were pooled per sample). Control CFA alone plus PBS mice were sacrificed on day 40. The CNS mononuclear cells were isolated and immunostained for the surface markers CD45.2 and CD11b, and intracellular Jmjd3. (A)Representative CD45.2/CD11b dot plots with gates on $CD11b^{+}$ $CD45^{low}$ (resting microglia) and $CD11b^{+}$ $CD45^{high}$ (activated microglia/macrophages) populations are shown on the right. Representative overlays of Jmjd3 fluorescence histograms with the mean fluorescence intensity values of non stained (NS) cells, secondary antibody only stained cells, resting microglia and activated microglia/macrophages are shown on the left. (B) The fold change in Mean Fluorescence Intensity (MFI) of Jmjd3 was calculated by dividing the activated microglia/macrophages population ($CD11b^{+}$ $CD45^{high}$) MFI by the resting microglia population ($CD11b^{+}$ $CD45^{low}$) MFI of each sample. Bars represent mean ± standard error. n=3 samples/group. *$p < 0.05$. **$p < 0.01$.

4.3. Discussion

Activation of microglia is crucial for the initiation and progression of MS (Ajami et al., 2011; Raivich and Banati, 2004; Rasmussen et al., 2007). As shown in chapter 3, *in vitro*, Jmjd3 is induced upon activation of microglia with IFNγ and after ligation of TLR receptors, which are all important players in the immunopathogenesis of MS and EAE (Bsibsi et al., 2002; Olson and Miller, 2004; Olsson et al., 1990; Prinz et al., 2006). Furthermore, this up-regulation is followed by a corresponding increase in Jmjd3 recruitment at the promoter of inflammation

related genes. These results led to the hypothesis that the gene expression program triggered upon microglial activation in MS may be regulated by this epigenetic enzyme, and therefore Jmjd3 expression was studied in EAE.

To be able to measure Jmjd3 expression at the protein level in samples from EAE mice, a technique that allowed the differentiation between resting and activated microglia using relatively low cell numbers was required. While it is not possible to discriminate these two microglial populations by immunohistochemistry, flow cytometric analysis has been used to distinguish resting from activated microglia based on their CD11b and CD45 expression (Becher and Antel, 1996; Sedgwick et al., 1991; Stevens et al., 2002). Since discriminating Jmjd3 expression levels in activated versus resting microglia was crucial for this study, it was decided that this flow cytometry method was the best option to distinguish the two subsets. Therefore, the suitability of polyclonal Jmjd3 antibodies for flow cytometry was tested. A polyclonal Jmjd3 antibody, kindly provided by Dr. Gioacchino Natoli produced the optimal results, detecting 61.7% of HA positive HEK 293T cells transfected with a pCMV-HA-*Jmjd3* plasmid. Since it was not sought to obtain absolute values of Jmjd3 expression, but rather relative differences in expression between microglial populations, this antibody was suitable for Jmjd3 detection by flow cytometry in EAE mice samples.

Early in EAE, before the onset of clinical signs, microglia get activated by the pro-inflammatory cytokines released by auto-reactive CNS infiltrating T cells (Ponomarev et al., 2005). In C57BL/6 mice with no disease, the vast majority of $CD11b^{+}$ CNS mononuclear cells consisted of $CD45^{low}$ resting microglia with only 9.3% having an activated phenotype. Comparatively, C57BL/6 MOG_{35-55} EAE mice showed a considerable 5.5 fold increase in the proportion of $CD45^{high}$ activated microglia/macrophages. This increase of activated $CD11b^{+}$ $CD45^{high}$ cells during EAE indicates the activation and differentiation of $CD45^{low}$ microglia into activated cells. It has been shown in bone marrow chimera and parabiosis experiments that

activated microglia proliferate and that, at the peak of EAE disease, around 40% of the cells considered phenotypically as macrophages in acute inflammatory lesions are actually activated microglia (Ajami et al., 2011; Ponomarev et al., 2005). However, activation of pre-existing resting microglia and proliferation of activated cells does not account for the entire increase observed in the $CD11b^{+}$ $CD45^{high}$ population, which also reflects the migration of peripheral macrophages into the CNS and their incorporation into the $CD45^{high}$ pool.

Interestingly, C57BL/6 naïve mice had small populations of $CD11b^{+}$ $CD45^{high}$ and $CD11b^{-}$ $CD45^{+}$ cells. The existence of these two subsets in the normal murine CNS has been reported previously. The $CD11b^{+}$ $CD45^{high}$ cells correspond to CNS associated macrophages present in the perivascular space, meninges, subarachnoid space, and choroid plexus (Hickey and Kimura, 1988; Hickey et al., 1992; Sedgwick et al., 1991). The presence of $CD11b^{-}$ $CD45^{+}$ cells reflects the continuous immune surveillance by the peripheral immune system in the healthy CNS (Goverman, 2009; Sedgwick et al., 1991). However, it is also possible that additional cell activation may have occurred during *ex vivo* manipulation of the samples, although this would be comparable between all of the groups.

C57BL/6 mice immunised with CFA alone (which do not develop disease) showed a higher proportion of $CD11b^{+}$ $CD45^{high}$ activated cells than naïve mice. CFA contains mycobacterial PAMPs that bind to TLR2 and TLR4 receptors (Stenger and Modlin, 2002). Additionally, these control mice received intravenous injections of pertussis toxin which is also a TLR4 receptor ligand (Kerfoot et al., 2004). In EAE, TLR ligation activates microglial innate immune responses that contribute to the disruption of self tolerance and the development of disease (Waldner et al., 2004). Therefore this activated microglial population in control mice is most probably the result of CFA and pertussis toxin-mediated stimulation.

As observed previously in microglia activated *in vitro,* $CD45^{high}$ activated microglia/macrophages from the CNS of C57BL/6 MOG_{35-55} EAE mice with severe disease also

showed an increase in the expression of Jmjd3. However, this increase was of a lesser extent than the up-regulation observed in the *in vitro* experiments. This difference can be explained by the fact that, under the pathophysiological conditions present in the brain of EAE mice, microglia become activated by smaller amounts of cytokines or other innate immunity stimulants, while in the *in vitro* experiments, very potent pro-inflammatory ligands (such as LPS) at concentrations that resulted in the highest up-regulations were used.

In the previous chapter, stimulation of the TLR4 and TLR2 receptors was demonstrated to cause the highest *Jmjd3* up-regulation in primary microglia *in vitro.* The $CD45^{high}$ activated microglia/macrophages from the CNS of C57BL/6 mice immunised with CFA alone showed a discrete increase of Jmjd3 levels compared to the resting microglia population. Although this was not significant, it contrasts with the absence of Jmjd3 up-regulation in activated microglia/macrophages from naïve mice. These results suggest that CFA-dependent microglial activation, most probably through TLR2 and TLR4, is accompanied by induction of Jmjd3 *in vivo*.

The main objective of this study was to elucidate if the increased expression of Jmjd3 in microglia contributes towards the initiation and maintenance of the pathological, chronically activated phenotype observed during the progressive phase of MS and EAE. Therefore, having demonstrated that Jmjd3 is up-regulated in activated microglia in a severe EAE disease setting, a CR-EAE mouse model was used to determine if activated microglia isolated from mice in late remission showed any differences in the expression of Jmjd3 compared to microglia isolated from mice in the acute phase.

In the CNS mononuclear cells isolated from MOG_{35-55} CR-EAE Biozzi ABH mice at the disease onset and first relapse, the majority of $CD11b^{+}$ cells were $CD45^{high}$ activated microglia/macrophages. As discussed for the C57BL/6 EAE study, this increase is secondary to activation of $CD11b^{+}$ $CD45^{low}$ cells and to the migration of peripheral macrophages into the

CNS. As mice recovered from the first relapse and entered into the second remission, the percentage of activated Cd11b^{+} CD45^{high} cells decreased. This most likely reflects the exit of blood-derived myelomonocytic cells from the CNS as well as the apoptosis of a fraction of the proliferated resident microglia (Ajami et al., 2011). However, in samples from mice sacrificed during the second remission, the activated microglia/macrophage population remained significantly larger than in the CNS of naïve mice. This finding agrees with previous observations made in CR-EAE and progressive MS where microglia were shown to remain permanently activated after the acute inflammatory infiltrates had disappeared and the subjects did not show signs of active disease (Kutzelnigg et al., 2005; Rasmussen et al., 2007). Concomitantly, as the first relapse ended, the percentage of resting microglial cells also increased, which indicates that a proportion of expanded activated cells do revert to a resting stage once the inflammatory episode ceases, as previously described by Ajami *et al.* (Ajami et al., 2011). Most of the CNS CD11b^{+} cells in naïve and CFA alone control Biozzi ABH mice corresponded to resting microglia, with a small proportion being CD45^{high} cells. This subset was further enlarged in the latter control mice group most probably due to CFA-mediated TLR 2 and 4 stimulation, even though mice were taken 40 days post-immunisation.

Jmjd3 was up-regulated, both at the mRNA and protein levels, in activated microglia/macrophages from all of the different Biozzi ABH MOG_{35-55} CR-EAE disease stages studied. However, no differences in the degree of expression were observed between acute episodes of disease and second remission. This suggests that in EAE, microglia up-regulate Jmjd3, most probably immediately after becoming activated, as demonstrated *in vitro* in the previous chapter. However, the raised level of Jmjd3 expression does not increase further as a result of chronic activation. *In vitro* experiments showed that Jmjd3 is rapidly and intensively up-regulated shortly after microglial activation, and that the expression then go down considerably after 4 hours stimulation to levels which are close to basal state, but significantly

higher, even after 24 hours of continuous stimulation. Therefore, it is probable that this prolonged, relatively small up-regulation is what was detected in the activated microglia/macrophage samples analysed in this *in vivo* study. Additionally, Jmjd3 was also significantly increased in activated microglia/macrophages from mice immunised with CFA alone and in peritoneal macrophage samples from disease onset, again most probably secondary to CFA-mediated TLR dependent activation. Together, these results suggest that Jmjd3 induction in this model is not EAE dependent, but rather a general mechanism that occurs following activation of microglia by pro-inflammatory stimuli capable of inducing NF-κB signalling. However, the mRNA and protein levels of Jmjd3 were slightly increased in the second remissions samples compared to the CFA alone controls, which suggest an influence of disease specific mechanisms over the CFA-mediated stimulation in this further up-regulation of Jmjd3.

In conclusion, actively induced EAE is not the appropriate model to study the effect of disease dependent microglial activation in the expression of Jmjd3, since CFA peripheral administration appears to override disease-specific mechanisms by also causing activation of microglia. Other mouse models of MS that do not require administration of an adjuvant to propitiate disease induction would be more suited to study the up-regulation of Jmjd3 in relation with the initiation and maintenance of persistent microglial activation. The alternative models that could be used include passive induction of EAE through the adoptive transfer of myelin specific autoreactive T cells (Berger et al., 1997; Schluesener and Wekerle, 1985) and Theiler's murine encephalitis virus-induced demyelinating disease (Rodriguez et al., 1983; Tsunoda and Fujinami, 1996). This latter model would be ideal since it induces a chronic, $CD4^+$ T cell-mediated demyelinating disease with a clinical course and histopathology similar to that of chronic progressive MS, and would therefore allow the study of Jmjd3 expression in chronic activated microglia.

5. Results

5.1. Introduction

Microglial activation plays a central role during the acute and chronic phase of MS and EAE (Ajami et al., 2011; Raivich and Banati, 2004; Rasmussen et al., 2007). As the findings in chapters 3 and 4 showed that Jmjd3 is up-regulated in these cells upon activation, it was investigated whether knockdown or knockout of the gene had any functional consequences. This with the objective to elucidate how Jmjd3 up-regulation contributes to the induction and maintenance of the damaging, chronic microglial activation characteristic of the progressive stages of MS and EAE, and therefore whether Jmjd3 could be a potential therapeutic target.

In the first instance, *Jmjd3* knockdown was performed in microglia using recombinant lentiviral vector-mediated shRNA interference. Transfection of myelomonocytic lineage cells, including primary microglia, by standard methods produces very low transfection efficiencies and poor transfer of transgenes. When non-viral transfection methods are used, the exogenous genetic material enters the microglia and macrophages by endocytosis and is therefore susceptible to be digested by lysosomal nucleases (Burke et al., 2002). Since lentiviruses can infect and integrate into the genome of non-dividing cells (Naldini et al., 1996), lentiviral vectors have been successfully used for transgene delivery and expression in cells difficult to transfect, such as primary microglia (Balcaitis et al., 2005; Fang et al., 2009; Neumann and Takahashi, 2007; Tsuji et al., 2005). Exogenous genetic material is transferred to an organism via a virus in a process called transduction (Griffiths, 2005).

Lentiviral vectors are replication-defective retroviruses derived from human immunodeficiency virus (HIV)-1. The vesicular stomatitis virus glycoprotein (VSV-G) in the envelope membrane of lentiviral particles allows them to transduce a broad range of cell types, including primary cells (Naldini et al., 1996; Trono, 2002). Second generation lentiviral

vector systems consist of three plasmids: a packaging plasmid, an envelope plasmid, and a transfer vector (Trono, 2002). The transfer vector contains the transgene or silencing short hairpin RNAs (shRNAs) to be delivered, along with the cis-acting elements required for genomic RNA production and packaging (Tiscornia et al., 2006b). When these three plasmids are co-transfected into 293 T cells, viral particles accumulate in the supernatant and can be purified by ultracentrifugation (Barry et al., 2001).

RNA interference (RNAi) is a technique based on a naturally occurring post-transcriptional process in which a long double-stranded RNA (dsRNA) introduced into a cell or organism is processed to short interfering RNAs (siRNAs) or micro RNAs (miRNAs), which inhibit gene expression in a sequence-specific manner through a mRNA degradation pathway (Denli and Hannon, 2003; Elbashir et al., 2001). ShRNAs are synthetic genetically engineered analogs of siRNAs and miRNA precursor molecules that can target specific genes for silencing (McManus et al., 2002; Paddison et al., 2002). MicroRNA-adapted shRNAs (shRNAmirs) are shRNAs whose design is based on the endogenous microRNA mir-30, which results in an increase in Drosha and Dicer processing that in turn improves the knockdown efficiency (Boden et al., 2004; Silva et al., 2005). In order to knockdown *Jmjd3*, a set of four pre-designed *Jmjd3* shRNAmir constructs cloned into a pGIPZ transfer vector (Thermo Scientific Open Biosystems GIPZ Lentiviral shRNAmir Library) were used to produce lentiviral vectors in 293 T cells and then transduce primary microglia. The pGIPZ vector expresses GFP under the control of a cytomegalovirus (CMV) promoter to allow easy distinction of transduced cells.

Secondly, to determine the functional role of Jmjd3 *in vivo*, a *Jmjd3* knockout mouse model was also used. Burgold *et al*. generated *Jmjd3* knockout mice by injecting *Jmjd3* gene trap ES cells into C57BL/6 blastocycts. The gene trap cassette is inserted in the intron 1 of *Jmjd3* and consists of a splice acceptor, the β-galactosidase neomycin resistance gene fusion (as reporter/selectable genetic marker) and a transcriptional termination polyadenylation signal.

Integration of this cassette into the first intron produces a fusion transcript between the first exon of *Jmjd3* and the β-galactosidase neomycin resistance gene fusion. This fusion transcript is prematurely terminated at the polyadenylation site resulting in a truncated, non-functional transcript and thus in loss of Jmjd3. *Jmjd3*$^{-/-}$ mice are homozygous for the trapped allele, which causes complete blockage of *Jmjd3* expression. *Jmjd3* knockout causes neonatal lethality due to respiratory failure, since Jmjd3 is indispensable for the maintenance of the pre-Bötzinger complex, which is a cluster of neurons in the brainstem essential for the generation of respiratory rhythm in mammals. (Burgold et al., 2012). Therefore, in order to perform experiments with *Jmjd3*$^{-/-}$ knockout microglia, E18.5 embryos were used to prepare primary microglial cultures.

5.2. Results

5.2.1. Lentiviral Jmjd3 knockdown

To study the physiological function of Jmjd3 in primary microglia, lentiviral vectors expressing shRNAmirs were used to transduce microglia and block the expression of *Jmjd3*.

Lentiviral particles carrying pGIPZ transfer vectors with shRNAmirs directed either against *Jmjd3*, *Gapdh* (positive control) or a non-silencing control were produced in 293 T cells. The titer of the lentiviral preparations was determined by transducing 293 T cells as described in section 2.3.1 and ranged between 10^8 to 10^9 viral particles/ml, which are suitable values (Tiscornia et al., 2006b) and indicate the production and purification of the lentiviral vectors was adequate.

To test the ability of the lentiviral vectors to infect target cells as well as the knockdown efficiency of the shRNAmirs constructs being used, a mouse fibroblast cell line, L929, was employed in the first instance. L929 cells were transduced at a multiplicity of infection (MOI)

(i.e. transduction units per cell) of 50 with lentiviral vectors containing either non-silencing-shRNAmir, *Gapdh*-shRNAmir or one of four different *Jmjd3*-shRNAmirs. The cells were cultured for 48 hours with the lentiviral vectors and the GFP expression was then measured by flow cytometry to determine the transduction efficiency. To confirm gene expression knockdown, the mRNA of the transduced cells was analysed by qRT-PCR and compared to non-silencing and non-transduced experimental controls.

The transduction efficiency of all of the different shRNAmir-lentiviral vectors analysed was above 90% in L929 cells (Figure 5.1 A). As expected, the non-silencing control vector did not knockdown either *Gapdh* or *Jmjd3*. A successful knockdown is considered any value over and above 70% (Root et al., 2006; Trono, 2002). The *Gapdh*-shRNAmir control produced a knockdown of 72.3% (Figure 5.1 B). However, none of the shRNAmirs directed against *Jmjd3* generated a knockdown above 70% (Figure 5.1 C). This could mean that, while the transfection efficiency was high, the number of cells not expressing the *Jmjd3*-shRNAmir is still high enough to mask the knockdown. It could also indicate that the sequences of the shRNAmirs used are not able to produce degradation of the target mRNAs. These two possibilities could be overcome by separating high GFP expressing cells through flow cytometry sorting, by trying different shRNAmirs or by pooling shRNAmirs to accumulate their effects. However, the purpose of this experiment was to test the efficiency of the lentiviral vectors to transduce cells. As the efficiency of RNAi can vary between different cell types (Oberdoerffer et al., 2005), the knockdown of *Jmjd3* was not further optimised for L929 cells, but was then investigated using primary microglia.

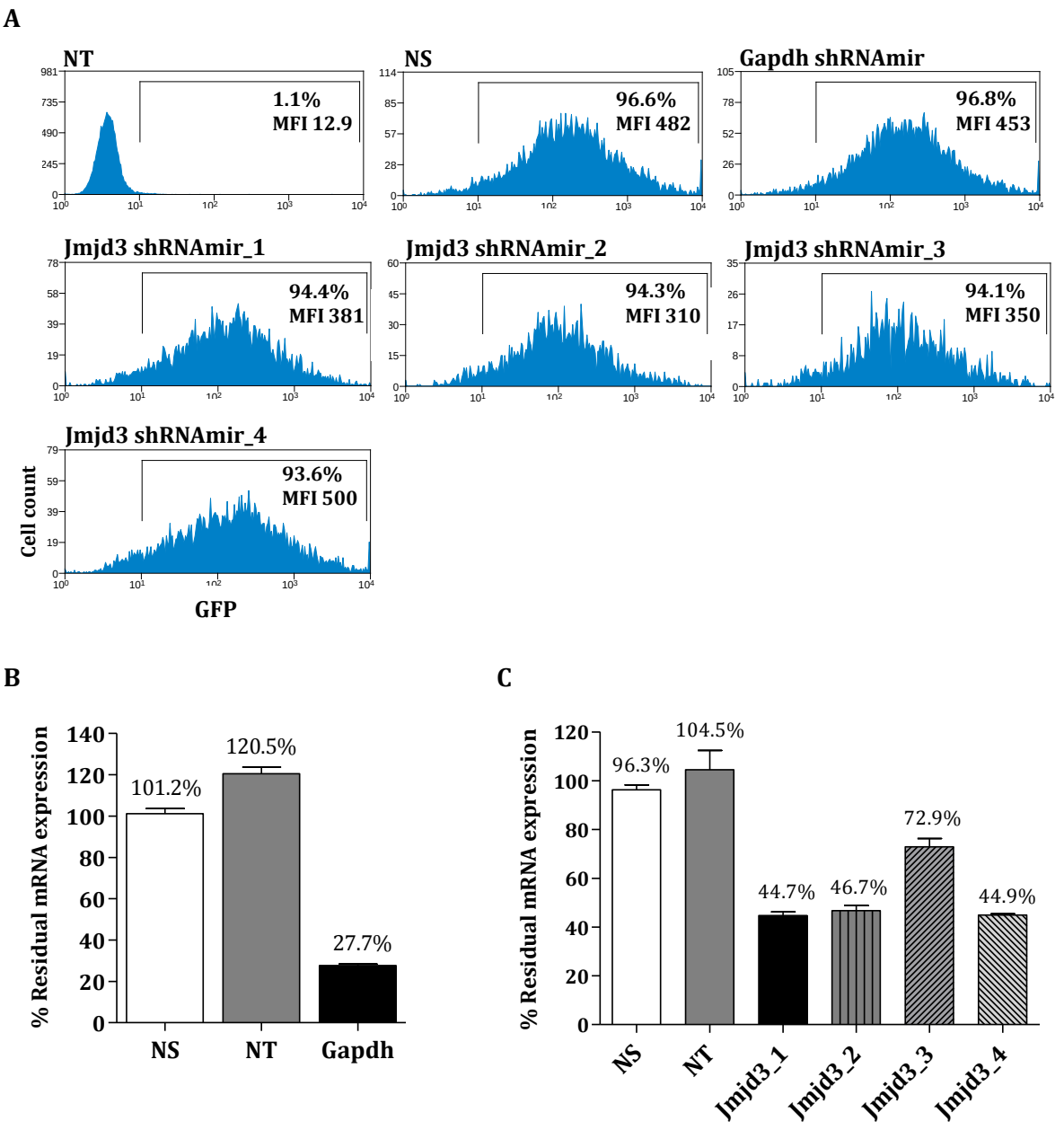

Figure 5.1. Lentiviral transduction of L929 cells. The cells were transduced with lentiviral particles containing either non-silencing-shRNAmir (NS), *Gapdh*-shRNAmir (A) or one of four different *Jmjd3*-shRNAmirs (B) at a MOI of 50 for 48 hours. (A) The transduction efficiency was analysed by flow cytometry for green fluorescent protein (GFP) fluorescence. Gates were set to exclude non-transduced (NT) cells. The percentage and the mean fluorescence intensity (MFI) of the transduced cells are shown per histogram. Representative experiment of 3. (B and C) The mRNA levels were measured by qRT-PCR and normalised to HPRT. Knockdown was calculated as the percentage of residual mRNA expression relative to the non-silencing control. NT= non-transduced control. Data are represented as mean ± standard error between triplicates.

To determine the optimal transduction conditions, primary microglia were transduced with lentiviral particles containing non-silencing-shRNAmir or *Gapdh*-shRNAmir at a MOI of 100, 250, 500 and 1000. Cells were cultured for 48 hours and GFP expression was then determined by flow cytometry as a measure of transduction.

The proportion of GFP positive cells increased from 19.4% with a MOI of 100 to 40.7% with a MOI of 250. However the percentage of transduced microglia did not increase much further with MOIs of 500 and 1000, achieving 43% and 45.5% transduction rates, respectively. The overall transduction rate as well as the MFI increased in an MOI-dependent manner. While the percentage of GFP-positive cells reached a plateau after a MOI of 250, the MFI did rise with MOIs of 500 and 1000 to 61.4 and 65.3 respectively. This increase in the MFI suggests that, in the transduced microglia, more than one copy of the pGIPZ vector integrated in the genome, resulting in increased GFP protein expression per cell (Figure 5.2).

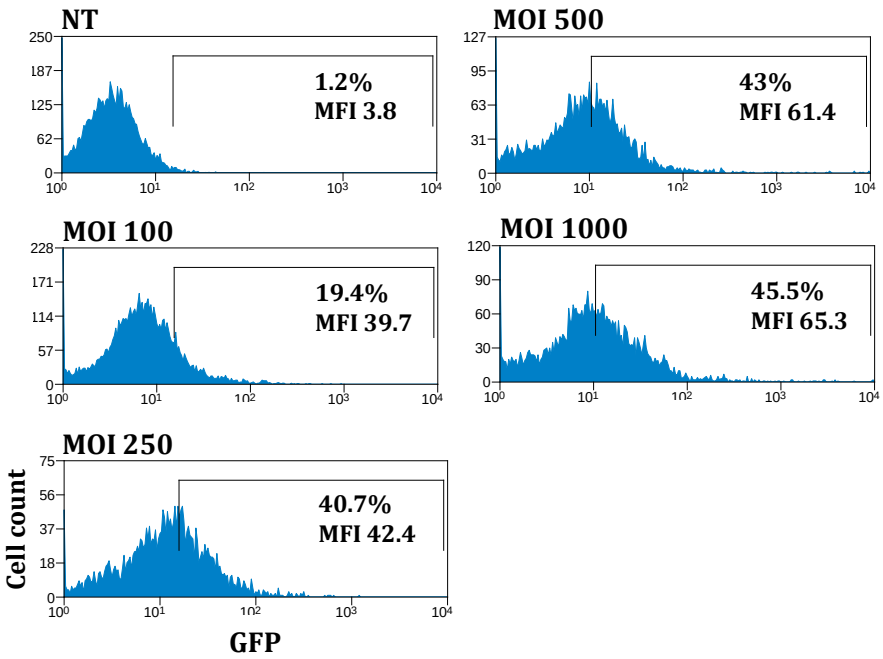

Figure 5.2. Lentiviral transduction efficiency in primary microglia. Primary murine microglial cells were transduced with lentiviral vectors at the multiplicities of infection (MOIs) indicated for 48 hours. Transduction efficiency was analysed by flow cytometry for green fluorescent protein (GFP) fluorescence. Gates were set against non-transduced (NT) cells. The percentage and the mean fluorescence intensity (MFI) of the transduced cells are shown per histogram. Representative experiment of 3.

In order to achieve a successful knockdown of the target gene, the transfection efficiency should be ideally higher than 70 percent (Trono, 2002). Given that this was not achieved, despite using a high titer of virus, the length of incubation time with the lentiviral particles was increased with the aim of achieving a higher transduction rate.

Primary cells were transduced with lentiviral particles at a MOI of 500 for 48 and 72 hours. However, transfection efficiency did not improve following 72 hours incubation with the pGIPZ shRNAmir lentiviral vectors in comparison to 48 hours, indicating that this was not the rate limiting factor (Figure 5.3).

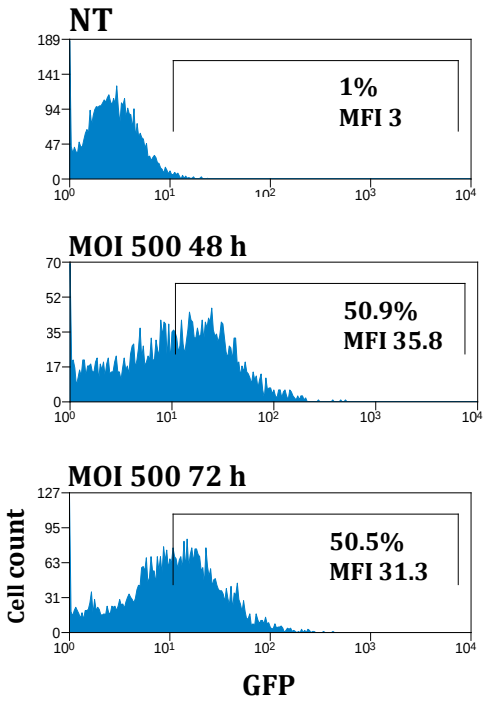

Figure 5.3. Effect of longer incubation time on lentiviral transduction efficiency in primary microglia. Primary murine microglial cells were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 500 for either 48 or 72 hours. Transduction efficiency was analysed by flow cytometry for green fluorescent protein (GFP) fluorescence. Gates were set against non-transduced (NT) cells. The percentage and the mean fluorescence intensity (MFI) of the transduced cells are shown per histogram. Representative experiment of 3.

Polybrene is a cationic polymer that is typically used to increase the efficiency of retroviral infection. Both the cell surface and the virus lipid membrane have a net-negative charge. Positively charged polybrene binds to cell surfaces and neutralises their charge. This facilitates early virus-cell interactions by counteracting repulsive electrostatic forces and promoting the binding of viral glycoproteins to their receptors on the cell membrane (Coelen et al., 1983; Davis et al., 2002). Consequently, primary microglia were incubated with polybrene for 12 hours before the addition of lentiviral particles at a MOI of 500 and further incubation for 48 hours.

The pre-incubation of primary microglia with polybrene only partially increased the overall transduction rate from 46% with lentiviral vectors alone to 50.1%, and the GFP fluorescence intensity from 30.3 to 39.3. Therefore polybrene addition failed to aid the achievement of satisfactory transduction in primary microglia (Figure 5.4).

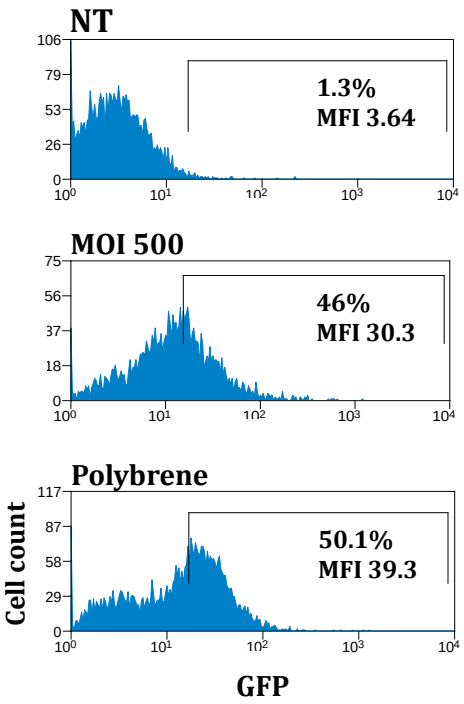

Figure 5.4. Polybrene does not sufficiently increase the efficiency of retroviral infection in primary microglia. Primary murine microglial cells were either just transduced with lentiviral vectors at a multiplicity of infection (MOI) of 500 for 48 hours or were pre-incubated with 8 µg/ml of polybrene for 12 hours before the addition of the lentiviral particles. Transduction efficiency was analysed by flow cytometry for green fluorescent protein (GFP) fluorescence. Gates were set to exclude non-transduced (NT) cells. The percentage and the mean fluorescence intensity (MFI) of the transduced cells are shown per histogram. Representative experiment of 3.

To increase the interaction between active lentiviruses and cells, the addition of mechanical methods (such as centrifugation) has previously been used to increase transduction efficiency (Bahnson et al., 1995; Kotani et al., 1994). However, when this approach was attempted the

primary microglial cells died, most likely due to them being unable to endure the process of being scraped, centrifuged with the lentiviral vectors and re-seeded. Additionally, primary microglia could not be cultured in plates suitable for centrifugation (such as 96-well plates) because the small well size would have not allowed the harvest of the cells for experiments, which was done by dislodging them with a cell scraper.

Magnetofection is a transduction method that associates gene vectors (such as lentiviruses) with magnetic nanoparticles coated with cationic molecules. A magnetic force is then applied to drag the lentivirus-nanoparticle complexes towards the cells to be transduced. This concentrates the lentiviruses near the target cells and eases their interaction. This results in higher transduction efficiencies, allows the transduction of cell types that are hard to infect (such as primary cells), reduces the transduction time and decreases the retroviral vector titer needed (Scherer et al., 2002). Given this, magnetofection was employed to transduce primary microglia, in order to attain satisfactory transduction efficiencies. Unfortunately, the use of magnetic transduction only increased the transduction rate moderately, and not to an adequate level to be able to deliver enough shRNAmirs to knock down gene expression to a sufficient degree (Figure 5.5).

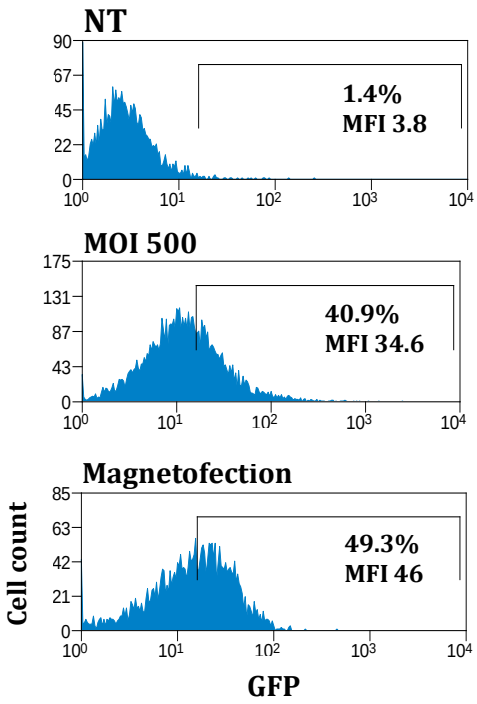

Figure 5.5. Magnetofection does not sufficiently increase the efficiency of retroviral infection in primary microglia. Primary murine microglial cells were either just transduced with lentiviral vectors at a multiplicity of infection (MOI) of 500 and incubated for 24 hours or were placed on top of a magnetic plate and incubated for 30 minutes with a mixture of magnetic nanoparticles plus lentiviral particles at a MOI of 500, after which the culture vessel was removed from the magnet and the cells were incubated for further 24 hours (without replacing the media). Transduction efficiency was analysed by flow cytometry for green fluorescent protein (GFP) fluorescence. Gates were set to exclude non-transduced (NT) cells. The percentage and the median fluorescence intensity (MFI) of the transduced cells are shown per histogram. Representative experiment of 3.

5.2.2. *Jmjd3*$^{-/-}$ mice

Primary microglia transduction with lentiviral vectors could not be achieved at a sufficiently high level to allow the successful knockdown of *Jmjd3* through shRNAmirs, despite the use of polybrene and magnetic particles to try to improve the infection of cells and the transduction efficiencies. Therefore, to determine the impact of Jmjd3 loss in microglial activation, *Jmjd3*$^{-/-}$ mice generated by gene trapping (kindly provided by Dr. Gioacchino Natoli, IFOM-IEO Campus, Milan, Italy) were used to prepare primary microglial cultures. The absence of *Jmjd3*

mRNA in *Jmd3*$^{-/-}$ microglial cultures was confirmed by qRT-PCR. As loss of Jmjd3 causes neonatal lethality due to respiratory failure, E18.5 embryos were used to prepare primary microglial cultures. Microglial cells from *Jmjd3*$^{+/+}$, *Jmjd3*$^{+/-}$ and *Jmjd3*$^{-/-}$ embryos were treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2, 4 and 8 hours.

Jmjd3$^{+/-}$ microglia showed approximately a third of the level of *Jmjd3* mRNA compared to WT microglia (6.8 fold induction in *Jmjd3*$^{+/-}$ microglia vs. 20.2 in *Jmjd3*$^{+/+}$ microglia after 2 hours stimulation, 4.5 fold induction in *Jmjd3*$^{+/-}$ microglia vs. 12.9 in *Jmjd3*$^{+/+}$ microglia after 4 hours stimulation and 4.2 fold induction in *Jmjd3*$^{+/-}$ microglia vs. 7.4 in *Jmjd3*$^{+/+}$ microglia after 8 hours stimulation). As expected, *Jmjd3* expression was ablated in *Jmjd3*$^{-/-}$ microglia (Figure 5.6).

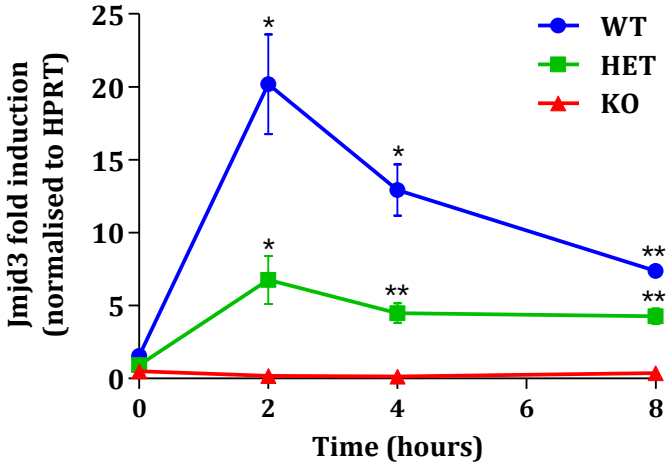

Figure 5.6. *Jmjd3* induction is ablated in *Jmjd3*$^{-/-}$ microglia. Primary microglial cultures from *Jmjd3*$^{+/+}$ (WT), *Jmjd3*$^{+/-}$ (HET) and *Jmjd3*$^{-/-}$ (KO) E18.5 embryos were treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2, 4 and 8 hours. The mRNA levels of *Jmjd3* were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non-stimulated. Data are represented as mean ± standard error between triplicates. *$p < 0.05$. **$p < 0.01$.

To initially investigate the functional effect of Jmjd3 absence on the pro-inflammatory activation of microglia, the gene expression of inflammatory mediators and effectors of the

innate immune response was determined in LPS plus IFNγ-treated *Jmjd3*$^{-/-}$ microglia compared to WT and heterozygous controls. The genes studied included *Ccl3*, *Ccl5*, *Ccl9*, *Cxcl11*, *Il6*, *Il12b*, *Nos2* and *TNFα*. These are significantly up-regulated in microglia following activation by pro-inflammatory stimuli (Figure 3.11), and are also important players in the immunopathogenesis of MS and EAE (Sospedra and Martin, 2005). Additionally, *Ccl3*, *Ccl5*, *Ccl9*, *Cxcl11*, *Il12b* and *Il6* have previously been described to be partially dependent on Jmjd3 for expression in *Jmjd3*$^{-/-}$ macrophages (De Santa et al., 2009). Primary microglial cultures from *Jmjd3*$^{+/+}$, *Jmjd3*$^{+/-}$ and *Jmjd3*$^{-/-}$ embryos were stimulated with LPS plus IFNγ, and the mRNA levels of the selected genes were measured by qRT-PCR.

No difference in the transcript levels of the immune response and inflammation related genes studied was observed between WT, heterozygous, and *Jmjd3* knockout microglia after stimulation for the different time points analysed (Figure 5.7).

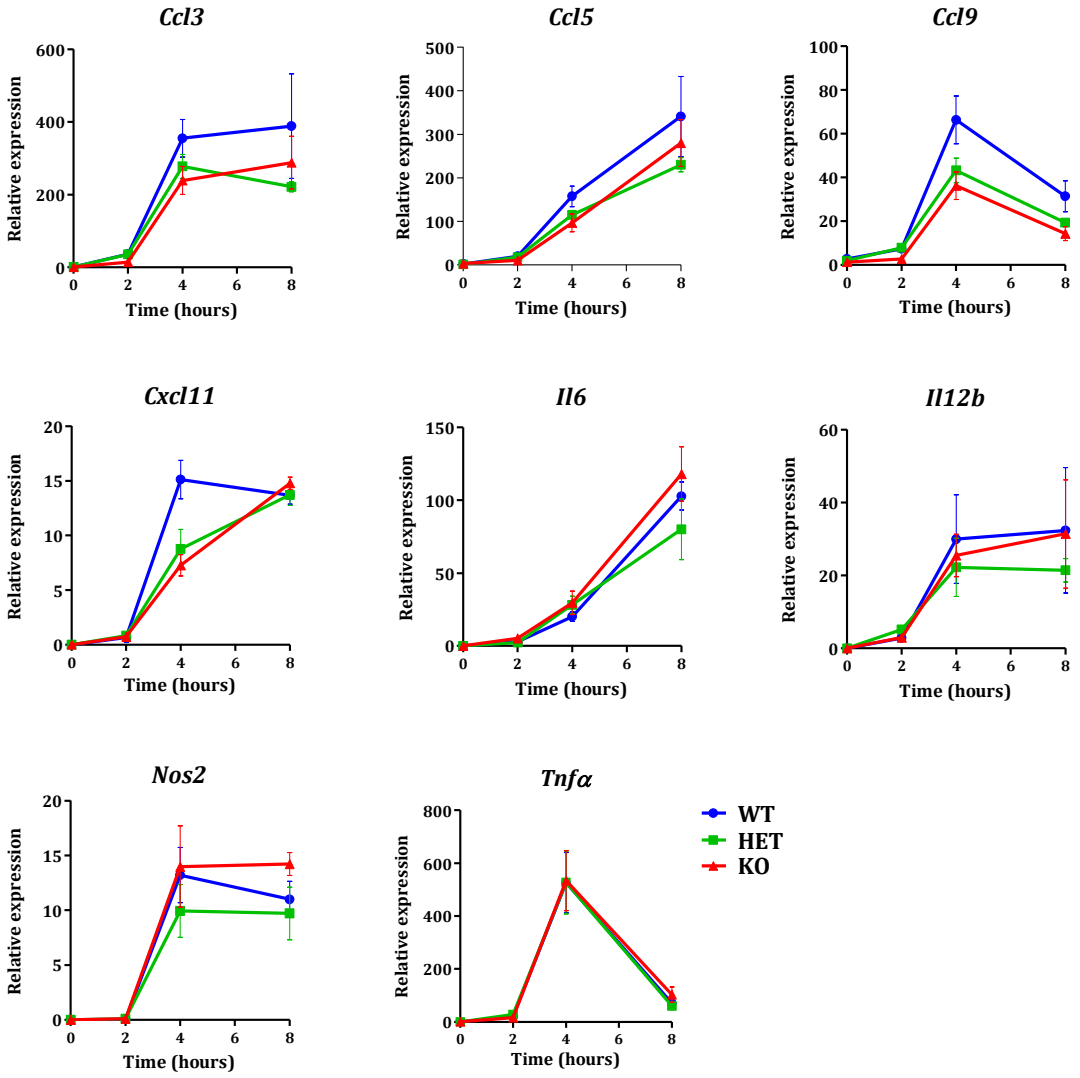

Figure 5.7. The expression of immune response and inflammation related genes in activated microglia is not dependent on Jmjd3. Primary microglial cultures from *Jmjd3*$^{+/+}$ (WT), *Jmjd3*$^{+/-}$ (HET) and *Jmjd3*$^{-/-}$ (KO) E18.5 embryos were treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 2, 4 and 8 hours. The mRNA levels were measured by qRT-PCR, normalised to *Hprt* and represented as the relative expression ($2^{-\Delta Ct}$). Data are represented as mean ± standard error between triplicates. All of the genes were significantly up-regulated above background levels with a p value < 0.05 after LPS plus IFNγ stimulation.

5.2.2.1. *Jarid2* expression in *Jmjd3*$^{-/-}$ microglia

Jarid2 is the only other JmjC-domain containing protein induced in primary microglia by pro-inflammatory stimuli. Jarid2 does not have histone methyltransferase activity, but it is a

regulator of histone methyltransferase complexes and promotes their recruitment to their target genes. In embryonic stem cells, Jarid2 associates with the PRC2 complex and stimulates its enzymatic activity resulting in increased levels of H3K27me3 and repression of transcription (Pasini et al., 2010). Since Jmjd3 up-regulation has been shown to reduce H3K27me3 levels at specific genes in certain cell types and *Jarid2* up-regulation occurred after the peak of *Jmjd3* expression, it was hypothesised that Jarid2 expression is increased in order to mediate the re-methylation of H3K27 after Jmjd3 induction. The possibility of an association between Jmjd3 levels and Jarid2 expression was explored by examining the mRNA levels of *Jarid2* by qRT-PCR in *Jmjd3* knockout microglia.

As shown in figure 5.8, the deletion of *Jmjd3* had no impact in the transcription of *Jarid2* in either resting or active microglia.

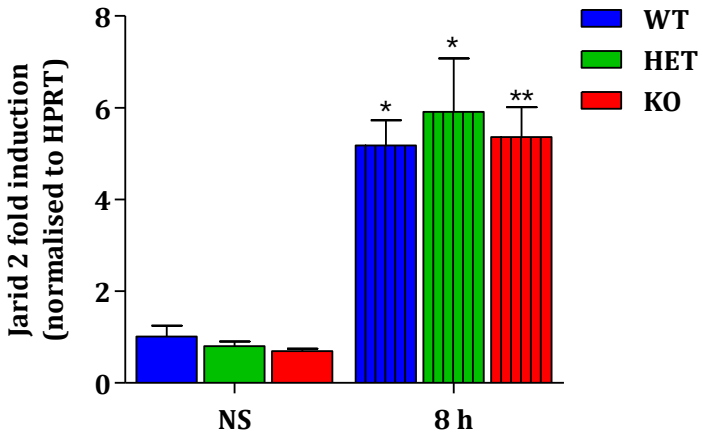

Figure 5.8. *Jarid2* expression is not affected in *Jmjd3* knockout activated microglia. Primary microglial cultures from *Jmjd3*$^{+/+}$ (WT), *Jmjd3*$^{+/-}$ (HET) and *Jmjd3*$^{-/-}$ (KO) E18.5 embryos were treated with 100 ng/ml of LPS plus 100 U/ml of IFNγ for 8 hours (corresponding to the peak of Jarid2 up-regulation). Jarid2 mRNA levels were measured by qRT-PCR, normalised to *Hprt* and represented as the fold induction over non-stimulated. Data are represented as mean ± standard error between triplicates. *$p < 0.05$. **$p < 0.01$.

Given that *Jmjd3* deletion had no repercussion in the expression of the examined effector genes in pro-inflammatory activated microglia, including genes that have been shown to be negatively regulated in *Jmjd3* knockout macrophages, this suggested that knockout of *Jmjd3* alone was not sufficient to induce a functional change to the microglia that was measurable in the parameters studied.

5.3. Discussion

In order to establish possible mechanisms through which Jmjd3 up-regulation upon pro-inflammatory stimulation of microglial could contribute to the persistent microglial activation observed in the progressive phase of MS and EAE, the functional repercussions of *Jmjd3* knockout were studied.

Initially, a lentiviral vector-mediated shRNA interference approach was tested to knockdown *Jmjd3* expression. Replication-defective lentiviral vectors are frequently used as carriers for gene or shRNA transfer into primary terminally differentiated cells difficult to transduce (Amado and Chen, 1999). Although the literature is limited, lentiviral vectors have been successfully used to transduce microglia. Balcaitis *et al*. reported transduction of microglia using a lentiviral-based vector system expressing GFP under the control of the murine stem cell virus promoter (MSCV). They achieved over 90% transduction efficiencies with the N9 and BV-2 microglial cell line. However, they also reported a maximum transduction efficiency of around 50% in primary microglia (Balcaitis et al., 2005), which is similar to the maximal efficiency achieved in this study. They attributed such discrepancy between the transduction efficiencies obtained in primary microglia and in microglial cell lines to differences in the proliferation rate between the two. Although lentiviral vectors are able to transduce non-proliferating cells efficiently, their integration to the target-cell genome is highly favoured by

cellular division (Naldini et al., 1996). After a lentiviral vector infects a cell, the vector RNA containing the shRNA is retro-transcribed into a double-stranded DNA molecule to then be transported to the nucleus where it integrates into the host genome (Amado and Chen, 1999; Buchschacher and Wong-Staal, 2000). Lentiviruses cannot efficiently transduce quiescent cells (G_0 state) because transition into the G_1b phase of the cell cycle is necessary for efficient, complete reverse transcription of the viral genome. In quiescent HIV-1 infected T cells, the retro-transcription of the viral genome is initiated, however, unlike in activated T cells, the reverse transcripts are incomplete and labile (Korin and Zack, 1998; Zack et al., 1990). Low levels of nucleotides in the resting cells partially explain the restricted HIV-1 replication, but other transcription factors and proteins present in partially activated cells are also needed for HIV transduction (Korin and Zack, 1999; Unutmaz et al., 1999; Zhang et al., 1999). The deficient replication of HIV-1 in resting T cells has also been attributed to the lack of ATP-dependant nuclear translocation (Bukrinsky et al., 1992) and a lack of integration of the pro-viral genome (Spina et al., 1995). Reduced and incomplete HIV-1 reverse transcription and transport to the nucleus has also been described in resting primary macrophages arrested early in the G_1 phase (Kootstra et al., 2000). Additionally, it has been reported that productive HIV infection of monocytes (with expression of the virus genes and production of viral particles) requires cell proliferation (Schuitemaker et al., 1994). Therefore, HIV-1 derived lentiviral vector-based transduction might only be effective in terminally differentiated target cells that progress through the cell cycle into G_1b. The need for activation of the cell cycle, but not necessarily progression to mitosis, for efficient lentiviral transduction has been described before in terminally differentiated cells other than T cells, such as hepatocytes (Park et al., 2000). Therefore, the low transduction efficiency in primary microglia could be explained because a proportion of cells in the primary microglial cultures were at a quiescent state. In primary T cells this transduction barrier has been overcome by pushing quiescent cells back

into the cell cycle by activating them via the T-cell receptor (TCR) or by cytokine stimulation (Korin and Zack, 1998; Unutmaz et al., 1999). However stimulation of microglia prior administration of the lentiviral vectors was not an option in this study, because it could have altered the epigenetic profile of the cells and possibly the physiological dynamics of Jmjd3 expression.

Another explanation as to why the different methods (higher MOI, polybrene and magnetic transduction) used to facilitate the introduction of the lentiviral particles inside the primary microglia failed to improve the transduction efficiency could be that, even though the lentiviruses gained access inside the cells and integrated into the genome, the shRNAmir-pGIPZ vectors were not expressed at optimal levels. The reason for this could be that the CMV promoter in the pGIPZ vector is not as active in primary microglia. Even though the CMV promoter is strong and constitutively expressed, it has been described it is not very active in mouse T and B cells (Lois et al., 2001). One more possibility is site of integration positional effects. Transfer vectors integrated in a silent chromatin region will show reduced or no expression (Emery et al., 2000). However this explanation is the least likely one since a high MOI was used and most probably more than one copy of the vector was integrated in the microglial genome.

A residual gene expression of around 5 to 15% of WT expression levels usually remains even after silencing with the most efficient shRNAs. This could constitute a problem when trying to block the expression of certain proteins, given that in some cases only 5% of WT expression levels is enough to preserve the protein function and a phenotype secondary to knockdown of expression is not observed (Tiscornia et al., 2006a). Because of this, and also because the *Jmjd3* knockout mice had become available for this study, the optimisation of the transduction of primary microglia using transfer vectors with different promoters was not attempted further.

To determine the functional impact of Jmjd3 absence on the gene expression program triggered by pro-inflammatory stimuli, the level of expression of a subset of inducible immune response and inflammation related genes was compared between WT, heterozygous, and *Jmjd3* knockout primary microglia treated with LPS plus IFNγ. The expression of all of the studied genes, before and after stimulation, was comparable between the three groups of samples. The fact that the expression of the Jmjd3 target genes *Ccl3*, *Ccl5*, *Il6* and *Nos2*, as demonstrated in the chapter 3, did not appear to be affected by *Jmjd3* deletion suggests either that Jmjd3 does not play any role in the regulation of their expression during microglial activation or that there are mechanisms providing functional redundancy. Under the assumption that Jmjd3 acts as a H3K27 demethylase to favour the expression of specific genes during pro-inflammatory microglial activation, the lack of effects on target genes in *Jmjd3* knockout microglia might suggest that other demethylases such as Utx (or other yet unidentified demethylases) compensate for the loss of Jmjd3.

Based on the findings of different groups, Jmjd3 may be involved in the regulation of transcription in several ways, including demethylation of H3K27me3 (Ishii et al., 2009; Lee et al., 2012; Miller et al., 2010), chromatin remodelling (Miller et al., 2010) and by assisting transcriptional elongation (Chen et al., 2012; De Santa et al., 2009). Although Utx's function in the regulation of transcription has been considered to be important mainly during development, in adult *Drosophila melanogaster* Utx colocalises with the elongating form of RNA pol II (Smith et al., 2008), which has also been described for Jmjd3 in macrophages and 293 T cells (Chen et al., 2012; De Santa et al., 2009). This indicates that Utx might have an active, similar role to Jmjd3 in ongoing transcription, and gives another argument to support the notion that the constitutively expressed Utx could compensate for the lack of Jmjd3.

Independently of its actual mechanism of action in the transcription process, Jmjd3 appears to be a transcriptional co-regulator of the activator type. In contrast to transcription factors, co-

regulators interact with the transcriptional machinery to favour transcription, but they only induce small transcriptional changes and are not indispensable for the expression of specific genes. This can be explained because there are thousands of different regulatory proteins and it is the sum of the action of all of them which influences the final outcome of transcription, rather than their individual effects. When one of the co-regulator proteins is missing, another or others co-regulators come in to play to compensate for its absence (Qiu et al., 2004; Roeder, 2005; Weake and Workman, 2010). Therefore, it appears that the up-regulation of Jmjd3 upon microglial activation constitutes an aid for transcription and compensatory/redundant pathways enter into play when it is missing.

Of the inducible immune response and inflammation related genes analysed in this study, the expression of *Ccl3*, *Ccl5*, *Ccl9*, *Cxcl11*, *Il12b* and *Il6* has been previously described to be affected in *Jmjd3* knockout murine foetal liver-derived macrophages stimulated with LPS plus IFNγ (De Santa et al., 2009). Satoh *et al*. also reported that the expression of *Il6* and *Il12b* was partially impaired in LPS treated *Jmjd3* knockout M-CSF murine bone marrow derived macrophages (Satoh et al., 2010). The contradictory findings between the previous reports in macrophages and this study highlights the fact that, despite sharing a myeloid origin, macrophages and microglia constitute distinct cell types (Ginhoux et al., 2010). They also suggest that Jmjd3 has different functions across cell types or that the mechanisms of transcription regulation by epigenetic enzymes are different between cell types. Another possibility is that under physiological circumstances the role of Jmjd3 is conserved but that the experimental conditions, for example the methods used to generate mature primary macrophages and microglia, caused changes in the gene expression program and the epigenetic regulation in the cells.

The mRNA stability of NF-kB induced genes plays an important role in regulating the kinetics of NF-kB-dependent gene expression. For example, if the mRNA of an NF- kB induced gene is

degraded very slowly, the expression level of the gene will be maintained for a long period after the termination of transcription (Sen and Smale, 2010). Hao and Baltimore reported that genes induced by pro-inflammatory stimuli (such as TNFα and LPS) can be divided in to three groups according to their expression kinetics. Genes in the third group (such as Ccl5 and TNFα) show a gradual, delayed transcription, but have very stable mRNAs that show very slow decrease in their levels even after withdrawal of the stimulus. Therefore, stability of inducible mRNAs is an important determinant of mRNA levels and in some cases overrides the effect of other elements controlling transcription (Hao and Baltimore 2009). Hence, an alternative possible reason why no differences were observed in the expression level of the genes analysed in this study is that the negative changes in the transcription process caused by the loss of Jmjd3 were masked by the effects of mRNA stability.

In conclusion, in activated microglia Jmjd3 acts as a transcriptional co-regulator and as such it facilitates transcription, but it is not essential, for the expression of specific genes. This is most likely due to the presence of compensatory/redundant co-regulators or mechanisms that enter into play to ensure the transcription of important effector genes remains unaffected.

6. General discussion

In the later stage of MS, the acute CNS inflammation produced by infiltrating T lymphocytes and macrophages is replaced by chronic, generalised inflammation caused by widespread persistent microglial activation and neurotoxicity. This leads to the progressive neurological disability characteristic of the late stages of the disease (Kutzelnigg et al., 2005). The mechanisms involved in the perpetuation of microglial activation are not fully understood and their elucidation is important for the development of better treatments for secondary progressive MS. Epigenetic mechanisms allow terminally differentiated cells to change their gene expression profiles in order to adapt to external cues (Jirtle and Skinner, 2007). In macrophages, Jmjd3 has been shown to be involved in the transcriptional process started by pro-inflammatory stimuli (De Santa et al., 2009; De Santa et al., 2007). This project analysed the possible contribution of Jmjd3 up-regulation upon microglial activation, to the initiation and maintenance of the chronic, pathologically activated phenotype seen during remissions and the progressive stage of MS and EAE.

This study has demonstrated that Jmjd3 is rapidly and strongly induced in primary microglia after exposure to pro-inflammatory cytokines and TLR ligands. The newly synthesised Jmjd3 is then recruited to the promoter region of *Il6*, *Ccl3*, *Ccl5* and *Nos2*, which are up-regulated in activated microglia. These genes subsequently presented a decrease in their H3K27me3 levels. This suggests that Jmjd3 might be involved in the regulation of the expression of such target genes, possibly by controlling the levels of H3K27 trimethylation (although other potential mechanisms of action cannot be ruled out) in response to external stimuli. These findings also led to the hypothesis that the microglial phenotype in MS and EAE may be controlled by dynamic epigenetic mechanisms. Jmjd3 was shown to be up-regulated in activated microglia/macrophages from mice in the acute and late phase of EAE. However, microglial activation accompanied by Jmjd3 induction was also observed in control mice

immunised with CFA alone. This indicated a CFA-mediated TLR2 and TLR4 stimulation of microglia, which could have overridden a potential disease-specific contribution to Jmjd3 upregulation. However, the fact that the second remission samples showed a slight increase in the expression of Jmjd3 compared to the control mice raised the possibility that this further up-regulation could be due to disease-associated mechanisms. This could be investigated in different EAE models where CFA is not used for induction of disease.

Previous reports in macrophages showed that the expression of a subset of immune response related genes was partly dependent on Jmjd3 induction in pro-inflammatory activated macrophages (De Santa et al., 2009; Satoh et al., 2010). Conversely, the deletion of *Jmjd3* in microglia did not have an effect on the expression of those, and other immune response and inflammation related genes analysed in this study, either before or after administration of LPS and IFNγ. This discrepancy in the role of Jmjd3 in regulating the gene expression program following activation between macrophages and microglia could derive from differences in the mechanisms of epigenetic regulation between cell types. However, the potential influence of the different experimental conditions used between studies in the function of Jmjd3 cannot be excluded.

The fact that *Ccl3*, *Ccl5*, *Il6* and *Nos2* showed Jmjd3 enrichment in their promoters following microglial activation, but did not show any effect in their gene expression levels in *Jmjd3* knockout microglia, suggests that there are mechanisms providing functional redundancy. Histone modifying enzymes are transcriptional co-regulators. At any one time, there are several co-activators acting in the regulatory region and promoter of a gene in order to promote transcription; this is the reason why individual transcriptional co-regulators have mainly modest effects on transcription (Alberts, 2008; Roeder, 2005). The role of Jmjd3 in transcription is that of a co-activator, either acting as an H3K27me3 demethylase (Ishii et al., 2009; Lee et al., 2012; Miller et al., 2010), aiding chromatin remodelling (Miller et al., 2010) or

contributing to transcriptional elongation, (Chen et al., 2012; De Santa et al., 2009) and as such, perhaps its activity can be fulfilled by other similar proteins. Utx belongs to the same sub-family of JmjC-domain containing proteins as Jmjd3 (Kdm6 demethylases), has also been demonstrated to be a H3K27me3 demethylase (Agger et al., 2007) and in *Drosophila melanogaster* it also co-localises with the elongating form of RNA pol II (Smith et al., 2008). Therefore, Utx might compensate for the deletion of Jmjd3 and this could be the reason why no differences could be observed in the expression of the immune response and inflammation related genes examined in *Jmjd3*$^{-/-}$ microglia in this study. In macrophages, Utx has been described to be a "housekeeping" histone demethylase, which is expressed at constant, low levels even after activation (De Santa et al., 2007). In this study, *Utx*'s expression also remained constantly low in activated microglia and it did not increase in *Jmjd3* knockout microglia following stimulation with LPS and IFNγ. Therefore, if Utx is compensating for the loss of Jmjd3, this data would suggest that the constitutively expressed low levels of the enzyme are sufficient to promote transcription of target genes.

In line with this, a recent study reported that a novel inhibitor of the Kdm6 subfamily of demethylases abrogated the LPS-induced TNFα production in human primary macrophages by blocking both Jmjd3 and Utx and preventing the demethylation of H3K27me3 in the TSS of the gene. When *Jmjd3* or *Utx* were knocked down individually using siRNAs, no changes in the expression of TNFα were observed. However, when both Jmjd3 and Utx were knocked down together, or their function was blocked with the novel inhibitor, the production of TNFα following stimulation was almost eliminated (Kruidenier et al., 2012). These findings suggest the possibility that, as in human macrophages, there is functional redundancy within the Kdm6 subfamily in primary microglia and that the combined H3K27me3 demethylase enzymatic activity of Jmjd3 and Utx might also be critical for the pro-inflammatory gene activation in this cell type.

Collectively, the findings in this thesis demonstrate that microglia undergo variations in the expression level of Jmjd3 as well as in the repressive epigenetic mark H3K27me3. These events, potentially together with the constitutively expressed Utx, might play a role in the initiation and maintenance of a pro-inflammatory gene expression program that causes the pathological persistent microglial activation state found in the late stages of MS and EAE. The presence of a combined function for Jmjd3 and Utx in the regulation of genes induced by pro-inflammatory stimuli in primary microglia remains to be proven. The up-regulation of Jmjd3 in activated microglia/macrophages by MS/EAE specific disease pathways in a CFA-free EAE model also awaits to be corroborated. Nevertheless, the role of disease-mediated mechanisms in the increase of Jmjd3 observed in activated microglia from the CNS of acute and CR-EAE mice in this study cannot be disregarded. In the MS mouse models used in this thesis, CFA could be acting as a surrogate for environmental factors (such as infections), which have been demonstrated to have an important impact on the instigation of relapses (Andersen et al., 1993; Buljevac et al., 2002). However, the importance of the putative combined effect of Jmjd3 and Utx in the regulation of gene expression, as well as the precise mechanisms by which they act (H3K27me3 demethylation and/or elongation of transcription) in microglia, should be determined before moving ahead with further animal studies.

It would also be relevant to examine the expression of Jmjd3, Utx and H3K27me3 in microglia and macrophages in normal versus EAE CNS using immunohistochemistry to then move to MS tissue.

The development of novel histone demethylase inhibitors is currently in progress (Cole, 2008; Spannhoff et al., 2009), as exemplified by the recent release of the Jmjd3 and Utx inhibitor GSK-J4 (Kruidenier et al., 2012). However, toxicological and pharmacological studies will need to be carried out before they can be safely used in mice. Another option to block histone-modifying enzymes *in vivo* is the CNS targeted delivery of siRNAs in complex with the rabies

virus glycoprotein (RVG)-9dR peptide, which binds specifically to the α7 subunit of the acetylcholine receptor in microglia and macrophages. These siRNA-RVG-9dRs have already been used in mice to block the production of TNFα specifically in CNS macrophages and microglia, which is an advantage over the histone demethylases inhibitors that, up to now, cannot be targeted to just one cell type (Kim et al., 2010).

Manipulation of the microglial expression of H3K27me3 demethylases in EAE, through the use of novel inhibitors or by the targeted delivery of specific siRNAs to the CNS, could lead to a better understanding of the role of epigenetically regulated mechanisms in the pathophysiology of chronic microglial activation in progressive MS. In the future this could lead to the development of novel therapeutic interventions.

7. References

Adams, R.A., Bauer, J., Flick, M.J., Sikorski, S.L., Nuriel, T., Lassmann, H., Degen, J.L., and Akassoglou, K. (2007). The fibrin-derived γ377-395 peptide inhibits microglia activation and suppresses relapsing paralysis in central nervous system autoimmune disease. The Journal of Experimental Medicine *204*, 571-582.

Adams, R.D., and Kubik, C.S. (1952). The morbid anatomy of the demyelinative disease. Am J Med *12*, 510-546.

Agalioti, T., Chen, G., and Thanos, D. (2002). Deciphering the Transcriptional Histone Acetylation Code for a Human Gene. Cell *111*, 381-392.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered Recruitment of Chromatin Modifying and General Transcription Factors to the IFN-^{2} Promoter. Cell *103*, 667-678.

Agger, K., Cloos, P.A., Christensen, J., Pasini, D., Rose, S., Rappsilber, J., Issaeva, I., Canaani, E., Salcini, A.E., and Helin, K. (2007). UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature *449*, 731-734.

Agger, K., Cloos, P.A., Rudkjaer, L., Williams, K., Andersen, G., Christensen, J., and Helin, K. (2009). The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. Genes Dev *23*, 1171-1176.

Agger, K., Christensen, J., Cloos, P.A., and Helin, K. (2008). The emerging functions of histone demethylases. Curr Opin Genet Dev *18*, 159-168.

Ajami, B., Bennett, J.L., Krieger, C., McNagny, K.M., and Rossi, F.M.V. (2011). Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci *14*, 1142-1149.

Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W., and Rossi, F.M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat Neurosci *10*, 1538-1543.

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol *2*, 675-680.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen Recognition and Innate Immunity. Cell *124*, 783-801.

Alberts, B. (2008). Molecular biology of the cell, 5th edn (New York ; Abingdon, Garland Science).

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732-738.

Allan, S.M., Tyrrell, P.J., and Rothwell, N.J. (2005). Interleukin-1 and neuronal injury. Nat Rev Immunol *5*, 629-640.

Amado, R.G., and Chen, I.S. (1999). Lentiviral vectors--the promise of gene therapy within reach? Science *285*, 674-676.

Amor, S., Groome, N., Linington, C., Morris, M.M., Dornmair, K., Gardinier, M.V., Matthieu, J.M., and Baker, D. (1994). Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. The Journal of Immunology *153*, 4349-4356.

Amor, S., Smith, P.A., Hart, B., and Baker, D. (2005). Biozzi mice: of mice and human neurological diseases. J Neuroimmunol *165*, 1-10.

Andersen, O., Lygner, P.-E., Bergström, T., Andersson, M., and Vablne, A. (1993). Viral infections trigger multiple sclerosis relapses: a prospective seroepidemiological study. J Neurol *240*, 417-422.

Andersson, Å., Covacu, R., Sunnemark, D., Danilov, A.I., Dal Bianco, A., Khademi, M., Wallström, E., Lobell, A., Brundin, L., Lassmann, H.*, et al.* (2008). Pivotal Advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. Journal of Leukocyte Biology *84*, 1248-1255.

Andersson, M., Alvarez-Cermeño, J., Bernardi, G., Cogato, I., Fredman, P., Frederiksen, J., Fredrikson, S., Gallo, P., Grimaldi, L.M., Grønning, M.*, et al.* (1994). Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report. Journal of Neurology, Neurosurgery & Psychiatry *57*, 897-902.

Anderton, J.A., Bose, S., Vockerodt, M., Vrzalikova, K., Wei, W., Kuo, M., Helin, K., Christensen, J., Rowe, M., Murray, P.G.*, et al.* (2011). The H3K27me3 demethylase, KDM6B, is induced by Epstein-Barr virus and over-expressed in Hodgkin's Lymphoma. Oncogene *30*, 2037-2043.

Ando, D.G., Clayton, J., Kono, D., Urban, J.L., and Sercarz, E.E. (1989). Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol *124*, 132-143.

Ascherio, A., and Munger, K.L. (2007). Environmental risk factors for multiple sclerosis. Part I: The role of infection. Annals of Neurology *61*, 288-299.

Bahnson, A.B., Dunigan, J.T., Baysal, B.E., Mohney, T., Atchison, R.W., Nimgaonkar, M.T., Ball, E.D., and Barranger, J.A. (1995). Centrifugal enhancement of retroviral mediated gene transfer. J Virol Methods *54*, 131-143.

Baker, D., O'Neill, J.K., Gschmeissner, S.E., Wilcox, C.E., Butter, C., and Turk, J.L. (1990). Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. J Neuroimmunol *28*, 261-270.

Balashov, K.E., Khoury, S.J., Hafler, D.A., and Weiner, H.L. (1995). Inhibition of T cell responses by activated human CD8+ T cells is mediated by interferon-gamma and is defective in chronic progressive multiple sclerosis. J Clin Invest *95*, 2711-2719.

Balcaitis, S., Weinstein, J.R., Li, S., Chamberlain, J.S., and Moller, T. (2005). Lentiviral transduction of microglial cells. Glia *50*, 48-55.

Banati, R.B., Newcombe, J., Gunn, R.N., Cagnin, A., Turkheimer, F., Heppner, F., Price, G., Wegner, F., Giovannoni, G., Miller, D.H.*, et al.* (2000). The peripheral benzodiazepine binding site in the brain in multiple sclerosis: Quantitative in vivo imaging of microglia as a measure of disease activity. Brain *123*, 2321-2337.

Bannister, A.J., Schneider, R., and Kouzarides, T. (2002). Histone methylation: dynamic or static? Cell *109*, 801-806.

Baranzini, S.E., Mudge, J., van Velkinburgh, J.C., Khankhanian, P., Khrebtukova, I., Miller, N.A., Zhang, L., Farmer, A.D., Bell, C.J., Kim, R.W.*, et al.* (2010). Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature *464*, 1351-1356.

Barcellos, L.F., Sawcer, S., Ramsay, P.P., Baranzini, S.E., Thomson, G., Briggs, F., Cree, B.C.A., Begovich, A.B., Villoslada, P., Montalban, X.*, et al.* (2006). Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. Human Molecular Genetics *15*, 2813-2824.

Barry, S.C., Harder, B., Brzezinski, M., Flint, L.Y., Seppen, J., and Osborne, W.R. (2001). Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression. Hum Gene Ther *12*, 1103-1108.

Bartholomaus, I., Kawakami, N., Odoardi, F., Schlager, C., Miljkovic, D., Ellwart, J.W., Klinkert, W.E.F., Flugel-Koch, C., Issekutz, T.B., Wekerle, H.*, et al.* (2009). Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. Nature *462*, 94-98.

Bartosik-Psujek, H., Belniak, E., Mitosek-Szewczyk, K., Dobosz, B., and Stelmasiak, Z. (2004). Interleukin-8 and RANTES levels in patients with relapsing-remitting multiple sclerosis (RR-MS) treated with cladribine. Acta Neurol Scand *109*, 390-392.

Basu, A., Krady, J.K., Enterline, J.R., and Levison, S.W. (2002a). Transforming growth factor beta1 prevents IL-1beta-induced microglial activation, whereas TNFalpha- and IL-6-stimulated activation are not antagonized. Glia *40*, 109-120.

Basu, A., Krady, J.K., O'Malley, M., Styren, S.D., DeKosky, S.T., and Levison, S.W. (2002b). The type 1 interleukin-1 receptor is essential for the efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury. J Neurosci *22*, 6071-6082.

Baxter, A.G. (2007). The origin and application of experimental autoimmune encephalomyelitis. Nat Rev Immunol *7*, 904-912.

Becher, B., and Antel, J.P. (1996). Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia. Glia *18*, 1-10.

Belogurov, A.A., Jr., Kurkova, I.N., Friboulet, A., Thomas, D., Misikov, V.K., Zakharova, M.Y., Suchkov, S.V., Kotov, S.V., Alehin, A.I., Avalle, B.*, et al.* (2008). Recognition and degradation of myelin basic protein peptides by serum autoantibodies: novel biomarker for multiple sclerosis. J Immunol *180*, 1258-1267.

Berger, T., Rubner, P., Schautzer, F., Egg, R., Ulmer, H., Mayringer, I., Dilitz, E., Deisenhammer, F., and Reindl, M. (2003). Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med *349*, 139-145.

Berger, T., Weerth, S., Kojima, K., Linington, C., Wekerle, H., and Lassmann, H. (1997). Experimental autoimmune encephalomyelitis: the antigen specificity of T lymphocytes determines the topography of lesions in the central and peripheral nervous system. Lab Invest *76*, 355-364.

Bernard, C.C., and Carnegie, P.R. (1975). Experimental autoimmune encephalomyelitis in mice: immunologic response to mouse spinal cord and myelin basic proteins. J Immunol *114*, 1537-1540.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235-238.

Bettelli, E., Pagany, M., Weiner, H.L., Linington, C., Sobel, R.A., and Kuchroo, V.K. (2003). Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. J Exp Med *197*, 1073-1081.

Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T., and Bruck, W. (2000). Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain *123 (Pt 6)*, 1174-1183.

Bjartmar, C., Kidd, G., Mork, S., Rudick, R., and Trapp, B.D. (2000). Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. Ann Neurol *48*, 893-901.

Bjartmar, C., Kinkel, R.P., Kidd, G., Rudick, R.A., and Trapp, B.D. (2001). Axonal loss in normal-appearing white matter in a patient with acute MS. Neurology *57*, 1248-1252.

Blasi, F., Riccio, M., Brogi, A., Strazza, M., Taddei, M.L., Romagnoli, S., Luddi, A., D'Angelo, R., Santi, S., Costantino-Ceccarini, E.*, et al.* (1999). Constitutive expression of interleukin-1beta (IL-1beta) in rat oligodendrocytes. Biol Chem *380*, 259-264.

Block, M.L., Zecca, L., and Hong, J.S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci *8*, 57-69.

Boden, D., Pusch, O., Silbermann, R., Lee, F., Tucker, L., and Ramratnam, B. (2004). Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. Nucleic Acids Res *32*, 1154-1158.

Boettiger, A.N., and Levine, M. (2009). Synchronous and Stochastic Patterns of Gene Activation in the Drosophila Embryo. Science *325*, 471-473.

Booss, J., Esiri, M.M., Tourtellotte, W.W., and Mason, D.Y. (1983). Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. Journal of the Neurological Sciences *62*, 219-232.

Boulé, M.W., Broughton, C., Mackay, F., Akira, S., Marshak-Rothstein, A., and Rifkin, I.R. (2004). Toll-like Receptor 9–Dependent and –Independent Dendritic Cell Activation by Chromatin–Immunoglobulin G Complexes. The Journal of Experimental Medicine *199*, 1631-1640.

Boven, L.A., Montagne, L., Nottet, H.S.L.M., and De Groot, C.J.A. (2000). Macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES mRNA semiquantification and protein expression in active demyelinating multiple sclerosis (MS) lesions. Clinical & Experimental Immunology *122*, 257-263.

Bowie, A., and O'Neill, L.A. (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. Journal of Leukocyte Biology *67*, 508-514.

Brenneman, D.E., Schultzberg, M., Bartfai, T., and Gozes, I. (1992). Cytokine regulation of neuronal survival. J Neurochem *58*, 454-460.

Brenner, T., Brocke, S., Szafer, F., Sobel, R.A., Parkinson, J.F., Perez, D.H., and Steinman, L. (1997). Inhibition of nitric oxide synthase for treatment of experimental autoimmune encephalomyelitis. The Journal of Immunology *158*, 2940-2946.

Brentano, F., Schorr, O., Gay, R.E., Gay, S., and Kyburz, D. (2005). RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via toll-like receptor 3. Arthritis & Rheumatism *52*, 2656-2665.

Brown, A., McFarlin, D.E., and Raine, C.S. (1982). Chronologic neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. Lab Invest *46*, 171-185.

Brynedal, B., Duvefelt, K., Jonasdottir, G., Roos, I.M., Åkesson, E., Palmgren, J., and Hillert, J. (2007). <italic>HLA-A</italic> Confers an <italic>HLA-DRB1</italic> Independent Influence on the Risk of Multiple Sclerosis. PLoS One *2*, e664.

Bsibsi, M., Ravid, R., Gveric, D., and van Noort, J.M. (2002). Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol *61*, 1013-1021.

Buchschacher, G.L., Jr., and Wong-Staal, F. (2000). Development of lentiviral vectors for gene therapy for human diseases. Blood *95*, 2499-2504.

Bukrinsky, M.I., Sharova, N., Dempsey, M.P., Stanwick, T.L., Bukrinskaya, A.G., Haggerty, S., and Stevenson, M. (1992). Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A *89*, 6580-6584.

Buljevac, D., Flach, H.Z., Hop, W.C.J., Hijdra, D., Laman, J.D., Savelkoul, H.F.J., van der Meché, F.G.A., van Doorn, P.A., and Hintzen, R.Q. (2002). Prospective study on the relationship between infections and multiple sclerosis exacerbations. Brain *125*, 952-960.

Burgold, T., Spreafico, F., De Santa, F., Totaro, M.G., Prosperini, E., Natoli, G., and Testa, G. (2008). The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One *3*, e3034.

Burgold, T., Voituron, N., Caganova, M., Tripathi, Prem P., Menuet, C., Tusi, Betsabeh K., Spreafico, F., Bévengut, M., Gestreau, C., Buontempo, S.*, et al.* (2012). The H3K27 Demethylase

JMJD3 Is Required for Maintenance of the Embryonic Respiratory Neuronal Network, Neonatal Breathing, and Survival. Cell Reports *2*, 1244-1258.

Burke, B., Sumner, S., Maitland, N., and Lewis, C.E. (2002). Macrophages in gene therapy: cellular delivery vehicles and in vivo targets. Journal of Leukocyte Biology *72*, 417-428.

Burns, J., Rosenzweig, A., Zweiman, B., and Lisak, R.P. (1983). Isolation of myelin basic protein-reactive T-cell lines from normal human blood. Cell Immunol *81*, 435-440.

Butovsky, O., Landa, G., Kunis, G., Ziv, Y., Avidan, H., Greenberg, N., Schwartz, A., Smirnov, I., Pollack, A., Jung, S.*, et al.* (2006a). Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. J Clin Invest *116*, 905-915.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A.E., Pluchino, S., Martino, G., and Schwartz, M. (2006b). Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Mol Cell Neurosci *31*, 149-160.

Camelo, S., Iglesias, A.H., Hwang, D., Due, B., Ryu, H., Smith, K., Gray, S.G., Imitola, J., Duran, G., Assaf, B.*, et al.* (2005). Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. J Neuroimmunol *164*, 10-21.

Cannella, B., and Raine, C.S. (1995). The adhesion molecule and cytokine profile of multiple sclerosis lesions. Annals of Neurology *37*, 424-435.

Cardona, A.E., Huang, D., Sasse, M.E., and Ransohoff, R.M. (2006a). Isolation of murine microglial cells for RNA analysis or flow cytometry. Nat Protocols *1*, 1947-1951.

Cardona, A.E., Pioro, E.P., Sasse, M.E., Kostenko, V., Cardona, S.M., Dijkstra, I.M., Huang, D., Kidd, G., Dombrowski, S., Dutta, R.*, et al.* (2006b). Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci *9*, 917-924.

Carson, M.J., Reilly, C.R., Sutcliffe, J.G., and Lo, D. (1998). Mature microglia resemble immature antigen-presenting cells. Glia *22*, 72-85.

Casamassimi, A., and Napoli, C. (2007). Mediator complexes and eukaryotic transcription regulation: An overview. Biochimie *89*, 1439-1446.

Ciavatta, D.J., Yang, J., Preston, G.A., Badhwar, A.K., Xiao, H., Hewins, P., Nester, C.M., Pendergraft, W.F., Magnuson, T.R., Jennette, J.C.*, et al.* (2010). Epigenetic basis for aberrant upregulation of autoantigen genes in humans with ANCA vasculitis. The Journal of Clinical Investigation *120*, 3209-3219.

Coelen, R.J., Jose, D.G., and May, J.T. (1983). The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV. Arch Virol *75*, 307-311.

Cole, P.A. (2008). Chemical probes for histone-modifying enzymes. Nat Chem Biol *4*, 590-597.

Colombo, M., Dono, M., Gazzola, P., Roncella, S., Valetto, A., Chiorazzi, N., Mancardi, G.L., and Ferrarini, M. (2000). Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. J Immunol *164*, 2782-2789.

Coman, I., Aigrot, M.S., Seilhean, D., Reynolds, R., Girault, J.A., Zalc, B., and Lubetzki, C. (2006). Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis. Brain *129*, 3186-3195.

Compston, A., and Coles, A. (2008). Multiple sclerosis. Lancet *372*, 1502-1517.

Compston, A., and McAlpine, D. (2005). McAlpine's multiple sclerosis, 4th edn ([Philadelphia], Churchill Livingstone/Elsevier).

Craner, M.J., Hains, B.C., Lo, A.C., Black, J.A., and Waxman, S.G. (2004a). Co-localization of sodium channel Nav1.6 and the sodium-calcium exchanger at sites of axonal injury in the spinal cord in EAE. Brain *127*, 294-303.

Craner, M.J., Newcombe, J., Black, J.A., Hartle, C., Cuzner, M.L., and Waxman, S.G. (2004b). Molecular changes in neurons in multiple sclerosis: altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na+/Ca2+ exchanger. Proc Natl Acad Sci U S A *101*, 8168-8173.

Crawford, M.P., Yan, S.X., Ortega, S.B., Mehta, R.S., Hewitt, R.E., Price, D.A., Stastny, P., Douek, D.C., Koup, R.A., Racke, M.K.*, et al.* (2004). High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. Blood *103*, 4222-4231.

Cross, A.K., and Woodroofe, M.N. (1999). Chemokines induce migration and changes in actin polymerization in adult rat brain microglia and a human fetal microglial cell line in vitro. Journal of Neuroscience Research *55*, 17-23.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T.*, et al.* (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744-748.

Chao, C.C., Hu, S., Molitor, T.W., Shaskan, E.G., and Peterson, P.K. (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. The Journal of Immunology *149*, 2736-2741.

Chao, M.J., Herrera, B.M., Ramagopalan, S.V., Deluca, G., Handunetthi, L., Orton, S.M., Lincoln, M.R., Sadovnick, A.D., and Ebers, G.C. (2010). Parent-of-origin effects at the major histocompatibility complex in multiple sclerosis. Hum Mol Genet *19*, 3679-3689.

Chen, S., Ma, J., Wu, F., Xiong, L.-j., Ma, H., Xu, W., Lv, R., Li, X., Villen, J., Gygi, S.P.*, et al.* (2012). The histone H3 Lys 27 demethylase JMJD3 regulates gene expression by impacting transcriptional elongation. Genes & Development *26*, 1364-1375.

Dalpke, A.H., Schäfer, M.K.-H., Frey, M., Zimmermann, S., Tebbe, J., Weihe, E., and Heeg, K. (2002). Immunostimulatory CpG-DNA Activates Murine Microglia. The Journal of Immunology *168*, 4854-4863.

Dasgupta, S., Zhou, Y., Jana, M., Banik, N.L., and Pahan, K. (2003). Sodium phenylacetate inhibits adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice at multiple steps. J Immunol *170*, 3874-3882.

Davies, C.A., Loddick, S.A., Toulmond, S., Stroemer, R.P., Hunt, J., and Rothwell, N.J. (1999). The progression and topographic distribution of interleukin-1beta expression after permanent middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab *19*, 87-98.

Davis, H.E., Morgan, J.R., and Yarmush, M.L. (2002). Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem *97*, 159-172.

De Santa, F., Narang, V., Yap, Z.H., Tusi, B.K., Burgold, T., Austenaa, L., Bucci, G., Caganova, M., Notarbartolo, S., Casola, S*., et al.* (2009). Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. EMBO J *28*, 3341-3352.

De Santa, F., Totaro, M.G., Prosperini, E., Notarbartolo, S., Testa, G., and Natoli, G. (2007). The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell *130*, 1083-1094.

Dean, G. (1967). Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. BMJ *2*, 724-730.

Dean, G., and Elian, M. (1997). Age at immigration to England of Asian and Caribbean immigrants and the risk of developing multiple sclerosis. Journal of Neurology, Neurosurgery & Psychiatry *63*, 565-568.

Deckert-Schluter, M., Schluter, D., and Schwendemann, G. (1992). Evaluation of IL-2, sIL2R, IL-6, TNF-alpha, and IL-1 beta levels in serum and CSF of patients with optic neuritis. J Neurol Sci *113*, 50-54.

del Rio-Hortega, P. (1932). Cytology and Cellular Pathology of the Nervous System

Denli, A.M., and Hannon, G.J. (2003). RNAi: an ever-growing puzzle. Trends Biochem Sci *28*, 196-201.

Diestel, A., Aktas, O., Hackel, D., Häke, I., Meier, S., Raine, C.S., Nitsch, R., Zipp, F., and Ullrich, O. (2003). Activation of Microglial Poly(ADP-Ribose)-Polymerase-1 by Cholesterol Breakdown Products during Neuroinflammation: a Link between Demyelination and Neuronal Damage. The Journal of Experimental Medicine *198*, 1729-1740.

Doukas, J., and Pober, J.S. (1990). IFN-gamma enhances endothelial activation induced by tumor necrosis factor but not IL-1. J Immunol *145*, 1727-1733.

Ebert, S., Gerber, J., Bader, S., Mühlhauser, F., Brechtel, K., Mitchell, T.J., and Nau, R. (2005). Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination. Journal of Neuroimmunology *159*, 87-96.

Egloff, S., and Murphy, S. (2008). Cracking the RNA polymerase II CTD code. Trends in Genetics *24*, 280-288.

Ehrlich, L.C., Hu, S., Sheng, W.S., Sutton, R.L., Rockswold, G.L., Peterson, P.K., and Chao, C.C. (1998). Cytokine Regulation of Human Microglial Cell IL-8 Production. The Journal of Immunology *160*, 1944-1948.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev *15*, 188-200.

Emery, D.W., Yannaki, E., Tubb, J., and Stamatoyannopoulos, G. (2000). A chromatin insulator protects retrovirus vectors from chromosomal position effects. Proc Natl Acad Sci U S A *97*, 9150-9155.

Eriksson, C., Van Dam, A.M., Lucassen, P.J., Bol, J.G., Winblad, B., and Schultzberg, M. (1999). Immunohistochemical localization of interleukin-1beta, interleukin-1 receptor antagonist and interleukin-1beta converting enzyme/caspase-1 in the rat brain after peripheral administration of kainic acid. Neuroscience *93*, 915-930.

Fang, K.M., Yang, C.S., Sun, S.H., and Tzeng, S.F. (2009). Microglial phagocytosis attenuated by short-term exposure to exogenous ATP through P2X receptor action. J Neurochem *111*, 1225-1237.

Farez, M.F., Quintana, F.J., Gandhi, R., Izquierdo, G., Lucas, M., and Weiner, H.L. (2010). Toll-like receptor 2 and poly(ADP-ribose) polymerase 1 promote central nervous system neuroinflammation in progressive EAE. Nat Immunol *11*, 97-97.

Felts, P.A., Woolston, A.M., Fernando, H.B., Asquith, S., Gregson, N.A., Mizzi, O.J., and Smith, K.J. (2005). Inflammation and primary demyelination induced by the intraspinal injection of lipopolysaccharide. Brain *128*, 1649-1666.

Ferguson, B., Matyszak, M.K., Esiri, M.M., and Perry, V.H. (1997). Axonal damage in acute multiple sclerosis lesions. Brain *120 (Pt 3)*, 393-399.

Fisniku, L.K., Brex, P.A., Altmann, D.R., Miszkiel, K.A., Benton, C.E., Lanyon, R., Thompson, A.J., and Miller, D.H. (2008). Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. Brain *131*, 808-817.

Flugel, A., Berkowicz, T., Ritter, T., Labeur, M., Jenne, D.E., Li, Z., Ellwart, J.W., Willem, M., Lassmann, H., and Wekerle, H. (2001). Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. Immunity *14*, 547-560.

Fogdell-Hahn, A., Ligers, A., Grønning, M., Hillert, J., and Olerup, O. (2000). Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease. Tissue Antigens *55*, 140-148.

Ford, A.L., Goodsall, A.L., Hickey, W.F., and Sedgwick, J.D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. The Journal of Immunology *154*, 4309-4321.

Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature *447*, 972-978.

Franciosi, S., Choi, H.B., Kim, S.U., and McLarnon, J.G. (2005). IL-8 enhancement of amyloid-beta (Aβ1-42)-induced expression and production of pro-inflammatory cytokines and COX-2 in cultured human microglia. Journal of Neuroimmunology *159*, 66-74.

Freund, J., Stern, E.R., and Pisani, T.M. (1947). Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and Mycobacteria in water-in-oil emulsion. J Immunol *57*, 179-194.

Friese, M.A., and Fugger, L. (2005). Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain *128*, 1747-1763.

Frohman, E.M., Racke, M.K., and Raine, C.S. (2006). Multiple Sclerosis — The Plaque and Its Pathogenesis. New England Journal of Medicine *354*, 942-955.

Fuda, N.J., Ardehali, M.B., and Lis, J.T. (2009). Defining mechanisms that regulate RNA polymerase II transcription in vivo. Nature *461*, 186-192.

Gay, D., and Esiri, M. (1991). Blood-brain barrier damage in acute multiple sclerosis plaques. An immunocytological study. Brain *114 (Pt 1B)*, 557-572.

Gay, F.W., Drye, T.J., Dick, G.W., and Esiri, M.M. (1997). The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion. Brain *120 (Pt 8)*, 1461-1483.

Gehrmann, J., Gold, R., Linington, C., Lannes-Vieira, J., Wekerle, H., and Kreutzberg, G.W. (1993). Microglial involvement in experimental autoimmune inflammation of the central and peripheral nervous system. Glia *7*, 50-59.

Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y.C., Yoshida, M., Ding, H.A., Gimbrone, M.A., Jr., Luster, A.D., Luscinskas, F.W., and Rosenzweig, A. (1999). MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature *398*, 718-723.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R*., et al.* (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. Science *330*, 841-845.

Gold, R., Linington, C., and Lassmann, H. (2006). Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain *129*, 1953-1971.

Gondokaryono, S.P., Ushio, H., Niyonsaba, F., Hara, M., Takenaka, H., Jayawardana, S.T.M., Ikeda, S., Okumura, K., and Ogawa, H. (2007). The extra domain A of fibronectin stimulates murine mast cells via Toll-like receptor 4. Journal of Leukocyte Biology *82*, 657-665.

Goverman, J. (2009). Autoimmune T cell responses in the central nervous system. Nat Rev Immunol *9*, 393-407.

Grafodatskaya, D., Choufani, S., Ferreira, J.C., Butcher, D.T., Lou, Y., Zhao, C., Scherer, S.W., and Weksberg, R. (2010). EBV transformation and cell culturing destabilizes DNA methylation in human lymphoblastoid cell lines. Genomics *95*, 73-83.

Grant, P.A., Duggan, L., Côté, J., Roberts, S.M., Brownell, J.E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C.D., Winston, F.*, et al.* (1997). Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes & Development *11*, 1640-1650.

Greter, M., Heppner, F.L., Lemos, M.P., Odermatt, B.M., Goebels, N., Laufer, T., Noelle, R.J., and Becher, B. (2005). Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nat Med *11*, 328-334.

Griffiths, A.J.F. (2005). Introduction to genetic analysis, 8th edn (New York ; Basingstoke, W. H. Freeman).

Gronseth, G.S., and Ashman, E.J. (2000). Practice parameter: the usefulness of evoked potentials in identifying clinically silent lesions in patients with suspected multiple sclerosis (an evidence-based review): Report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology *54*, 1720-1725.

Hanisch, U.K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci *10*, 1387-1394.

Hao, S., and Baltimore, D. (2009). The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat Immunol *10*, 281-288.

Harkiolaki, M., Holmes, S.L., Svendsen, P., Gregersen, J.W., Jensen, L.T., McMahon, R., Friese, M.A., van Boxel, G., Etzensperger, R., Tzartos, J.S.*, et al.* (2009). T Cell-Mediated Autoimmune Disease Due to Low-Affinity Crossreactivity to Common Microbial Peptides. Immunity *30*, 348-357.

Hawkes, C.H. (2007). Smoking is a risk factor for multiple sclerosis: a metanalysis. Multiple Sclerosis *13*, 610-615.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099-1103.

Hayes, G.M., Woodroofe, M.N., and Cuzner, M.L. (1987). Microglia are the major cell type expressing MHC class II in human white matter. Journal of the Neurological Sciences *80*, 25-37.

Hayes, M.P., Freeman, S.L., and Donnelly, R.P. (1995). IFN-γ priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and mRNA stability. Cytokine *7*, 427-435.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Held, T.K., Weihua, X., Yuan, L., Kalvakolanu, D.V., and Cross, A.S. (1999). Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. Infect Immun *67*, 206-212.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K.*, et al.* (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Heppner, F.L., Greter, M., Marino, D., Falsig, J., Raivich, G., Hovelmeyer, N., Waisman, A., Rulicke, T., Prinz, M., Priller, J.*, et al.* (2005). Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat Med *11*, 146-152.

Hickey, W., and Kimura, H. (1988). Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science *239*, 290-292.

Hickey, W.F., Vass, K., and Lassmann, H. (1992). Bone marrow-derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. J Neuropathol Exp Neurol *51*, 246-256.

Hoek, R.M., Ruuls, S.R., Murphy, C.A., Wright, G.J., Goddard, R., Zurawski, S.M., Blom, B., Homola, M.E., Streit, W.J., Brown, M.H.*, et al.* (2000). Down-Regulation of the Macrophage Lineage Through Interaction with OX2 (CD200). Science *290*, 1768-1771.

Hofman, F.M., von Hanwehr, R.I., Dinarello, C.A., Mizel, S.B., Hinton, D., and Merrill, J.E. (1986). Immunoregulatory molecules and IL 2 receptors identified in multiple sclerosis brain. J Immunol *136*, 3239-3245.

Hong, S., Cho, Y.W., Yu, L.R., Yu, H., Veenstra, T.D., and Ge, K. (2007). Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci U S A *104*, 18439-18444.

Howell, O.W., Palser, A., Polito, A., Melrose, S., Zonta, B., Scheiermann, C., Vora, A.J., Brophy, P.J., and Reynolds, R. (2006). Disruption of neurofascin localization reveals early changes preceding demyelination and remyelination in multiple sclerosis. Brain *129*, 3173-3185.

Huseby, E.S., Liggitt, D., Brabb, T., Schnabel, B., Ohlen, C., and Goverman, J. (2001). A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. J Exp Med *194*, 669-676.

Husebye, H., Halaas, O., Stenmark, H., Tunheim, G., Sandanger, O., Bogen, B., Brech, A., Latz, E., and Espevik, T. (2006). Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. EMBO J *25*, 683-692.

Huynh, J.L., and Casaccia, P. (2013). Epigenetic mechanisms in multiple sclerosis: implications for pathogenesis and treatment. Lancet Neurol *12*, 195-206.

Ishii, M., Wen, H., Corsa, C.A.S., Liu, T., Coelho, A.L., Allen, R.M., Carson, W.F., Cavassani, K.A., Li, X., Lukacs, N.W.*, et al.* (2009). Epigenetic regulation of the alternatively activated macrophage phenotype. Blood *114*, 3244-3254.

Ishizu, T., Osoegawa, M., Mei, F.J., Kikuchi, H., Tanaka, M., Takakura, Y., Minohara, M., Murai, H., Mihara, F., Taniwaki, T.*, et al.* (2005). Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. Brain *128*, 988-1002.

Jack, C.S., Arbour, N., Manusow, J., Montgrain, V., Blain, M., McCrea, E., Shapiro, A., and Antel, J.P. (2005). TLR Signaling Tailors Innate Immune Responses in Human Microglia and Astrocytes. The Journal of Immunology *175*, 4320-4330.

Jackson, S.J., Lee, J., Nikodemova, M., Fabry, Z., and Duncan, I.D. (2009). Quantification of myelin and axon pathology during relapsing progressive experimental autoimmune encephalomyelitis in the Biozzi ABH mouse. J Neuropathol Exp Neurol *68*, 616-625.

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet *33*, 245-254.

Janson, P.C., Linton, L.B., Bergman, E.A., Marits, P., Eberhardson, M., Piehl, F., Malmstrom, V., and Winqvist, O. (2011). Profiling of CD4+ T cells with epigenetic immune lineage analysis. J Immunol *186*, 92-102.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jepsen, K., Solum, D., Zhou, T., McEvilly, R.J., Kim, H.-J., Glass, C.K., Hermanson, O., and Rosenfeld, M.G. (2007). SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature *450*, 415-419.

Jirtle, R.L., and Skinner, M.K. (2007). Environmental epigenomics and disease susceptibility. Nat Rev Genet *8*, 253-262.

Johnson, G.B., Brunn, G.J., and Platt, J.L. (2004). Cutting Edge: An Endogenous Pathway to Systemic Inflammatory Response Syndrome (SIRS)-Like Reactions through Toll-Like Receptor 4. The Journal of Immunology *172*, 20-24.

Jou, I., Lee, J.H., Park, S.Y., Yoon, H.J., Joe, E.-H., and Park, E.J. (2006). Gangliosides Trigger Inflammatory Responses via TLR4 in Brain Glia. The American Journal of Pathology *168*, 1619-1630.

Juedes, A.E., and Ruddle, N.H. (2001). Resident and Infiltrating Central Nervous System APCs Regulate the Emergence and Resolution of Experimental Autoimmune Encephalomyelitis. The Journal of Immunology *166*, 5168-5175.

Junker, A., Krumbholz, M., Eisele, S., Mohan, H., Augstein, F., Bittner, R., Lassmann, H., Wekerle, H., Hohlfeld, R., and Meinl, E. (2009). MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain *132*, 3342-3352.

Kabat, E.A., Wolf, A., and Bezer, A.E. (1947). The Rapid Production of Acute Disseminated Encephalomyelitis in Rhesus Monkeys by Injection of Heterologous and Homologous Brain Tissue with Adjuvants. J Exp Med *85*, 117-130.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-[beta]. Nat Immunol *9*, 361-368.

Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., and Prat, A. (2007). Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Med *13*, 1173-1175.

Kerfoot, S.M., Long, E.M., Hickey, M.J., Andonegui, G., Lapointe, B.M., Zanardo, R.C.O., Bonder, C., James, W.G., Robbins, S.M., and Kubes, P. (2004). TLR4 Contributes to Disease-Inducing Mechanisms Resulting in Central Nervous System Autoimmune Disease. The Journal of Immunology *173*, 7070-7077.

Kielian, T., Esen, N., and Bearden, E.D. (2005). Toll-like receptor 2 (TLR2) is pivotal for recognition of S. aureus peptidoglycan but not intact bacteria by microglia. Glia *49*, 567-576.

Kim, S.S., Ye, C., Kumar, P., Chiu, I., Subramanya, S., Wu, H., Shankar, P., and Manjunath, N. (2010). Targeted delivery of siRNA to macrophages for anti-inflammatory treatment. Mol Ther *18*, 993-1001.

Klose, R.J., and Bird, A.P. (2006). Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci *31*, 89-97.

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715-727.

Klose, R.J., and Zhang, Y. (2007). Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol *8*, 307-318.

Koch, M.W., Metz, L.M., and Kovalchuk, O. (2013). Epigenetic changes in patients with multiple sclerosis. Nat Rev Neurol *9*, 35-43.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K., and Iwakura, Y. (2006). IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol *177*, 566-573.

Kono, H., and Rock, K.L. (2008). How dying cells alert the immune system to danger. Nat Rev Immunol *8*, 279-289.

Kootstra, N.A., Zwart, B.M., and Schuitemaker, H. (2000). Diminished Human Immunodeficiency Virus Type 1 Reverse Transcription and Nuclear Transport in Primary Macrophages Arrested in Early G1 Phase of the Cell Cycle. Journal of Virology *74*, 1712-1717.

Korin, Y.D., and Zack, J.A. (1998). Progression to the G1b Phase of the Cell Cycle Is Required for Completion of Human Immunodeficiency Virus Type 1 Reverse Transcription in T Cells. Journal of Virology *72*, 3161-3168.

Korin, Y.D., and Zack, J.A. (1999). Nonproductive human immunodeficiency virus type 1 infection in nucleoside-treated G0 lymphocytes. J Virol *73*, 6526-6532.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., and Lassmann, H. (2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am J Pathol *157*, 267-276.

Kotani, H., Newton, P.B., 3rd, Zhang, S., Chiang, Y.L., Otto, E., Weaver, L., Blaese, R.M., Anderson, W.F., and McGarrity, G.J. (1994). Improved methods of retroviral vector transduction and production for gene therapy. Hum Gene Ther *5*, 19-28.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. Trends Neurosci *19*, 312-318.

Kruidenier, L., Chung, C.-w., Cheng, Z., Liddle, J., Che, K., Joberty, G., Bantscheff, M., Bountra, C., Bridges, A., Diallo, H.*, et al.* (2012). A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. Nature *488*, 404-408.

Kurtzke, J.F. (1993). Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev *6*, 382-427.

Kutzelnigg, A., Lucchinetti, C.F., Stadelmann, C., Bruck, W., Rauschka, H., Bergmann, M., Schmidbauer, M., Parisi, J.E., and Lassmann, H. (2005). Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain *128*, 2705-2712.

Lang, H.L.E., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P., Sondergaard, L., Svejgaard, A., Wucherpfennig, K.*, et al.* (2002). A functional and structural basis for TCR cross-reactivity in multiple sclerosis. Nat Immunol *3*, 940-943.

Lassmann, H., Bruck, W., and Lucchinetti, C. (2001). Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. Trends Mol Med *7*, 115-121.

Lee, J.-S., Shukla, A., Schneider, J., Swanson, S.K., Washburn, M.P., Florens, L., Bhaumik, S.R., and Shilatifard, A. (2007). Histone Crosstalk between H2B Monoubiquitination and H3 Methylation Mediated by COMPASS. Cell *131*, 1084-1096.

Lee, K., Na, W., Lee, J.Y., Na, J., Cho, H., Wu, H., Yune, T.Y., Kim, W.-S., and Ju, B.-G. (2012). Molecular mechanism of Jmjd3-mediated interleukin-6 gene regulation in endothelial cells underlying spinal cord injury. Journal of Neurochemistry *122*, 272-282.

Lee, S.C., Liu, W., Dickson, D.W., Brosnan, C.F., and Berman, J.W. (1993). Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. The Journal of Immunology *150*, 2659-2667.

Lehnardt, S., Lachance, C., Patrizi, S., Lefebvre, S., Follett, P.L., Jensen, F.E., Rosenberg, P.A., Volpe, J.J., and Vartanian, T. (2002). The Toll-Like Receptor TLR4 Is Necessary for Lipopolysaccharide-Induced Oligodendrocyte Injury in the CNS. The Journal of Neuroscience *22*, 2478-2486.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J., and Vartanian, T. (2003). Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proceedings of the National Academy of Sciences *100*, 8514-8519.

Lehnardt, S., Schott, E., Trimbuch, T., Laubisch, D., Krueger, C., Wulczyn, G., Nitsch, R., and Weber, J.R. (2008). A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J Neurosci *28*, 2320-2331.

Levin Li, M.K.L.R.M.V., and et al. (2003). MUltiple sclerosis and epstein-barr virus. JAMA *289*, 1533-1536.

Li, Y., Chu, N., Hu, A., Gran, B., Rostami, A., and Zhang, G.-X. (2007). Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia. Brain *130*, 490-501.

Liggett, T., Melnikov, A., Tilwalli, S., Yi, Q., Chen, H., Replogle, C., Feng, X., Reder, A., Stefoski, D., Balabanov, R.*, et al.* (2010). Methylation patterns of cell-free plasma DNA in relapsing-remitting multiple sclerosis. J Neurol Sci *290*, 16-21.

Lindberg, R.L., De Groot, C.J., Montagne, L., Freitag, P., van der Valk, P., Kappos, L., and Leppert, D. (2001). The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis. Brain *124*, 1743-1753.

Linington, C., Bradl, M., Lassmann, H., Brunner, C., and Vass, K. (1988). Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol *130*, 443-454.

Lister, R., Pelizzola, M., Kida, Y.S., Hawkins, R.D., Nery, J.R., Hon, G., Antosiewicz-Bourget, J., O/'Malley, R., Castanon, R., Klugman, S.*, et al.* (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature *471*, 68-73.

Lock, C., Hermans, G., Pedotti, R., Brendolan, A., Schadt, E., Garren, H., Langer-Gould, A., Strober, S., Cannella, B., Allard, J.*, et al.* (2002). Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat Med *8*, 500-508.

Lois, C., Refaeli, Y., Qin, X.-F., and Van Parijs, L. (2001). Retroviruses as tools to study the immune system. Current Opinion in Immunology *13*, 496-504.

Lovett-Racke, A.E., Trotter, J.L., Lauber, J., Perrin, P.J., June, C.H., and Racke, M.K. (1998). Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. J Clin Invest *101*, 725-730.

Lublin, F.D., Reingold, S.C., and Sclerosis*, N.M.S.S.A.C.o.C.T.o.N.A.i.M. (1996). Defining the clinical course of multiple sclerosis: Results of an international survey. Neurology *46*, 907-911.

Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol *47*, 707-717.

Lucchinetti, C.F., Bruck, W., and Lassmann, H. (2004). Evidence for pathogenic heterogeneity in multiple sclerosis. Ann Neurol *56*, 308.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Lund, B.T., Ashikian, N., Ta, H.Q., Chakryan, Y., Manoukian, K., Groshen, S., Gilmore, W., Cheema, G.S., Stohl, W., Burnett, M.E.*, et al.* (2004). Increased CXCL8 (IL-8) expression in Multiple Sclerosis. J Neuroimmunol *155*, 161-171.

Lund, S., Christensen, K.V., Hedtjärn, M., Mortensen, A.L., Hagberg, H., Falsig, J., Hasseldam, H., Schrattenholz, A., Pörzgen, P., and Leist, M. (2006). The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. Journal of Neuroimmunology *180*, 71-87.

Mack, C.L., Vanderlugt-Castaneda, C.L., Neville, K.L., and Miller, S.D. (2003). Microglia are activated to become competent antigen presenting and effector cells in the inflammatory environment of the Theiler's virus model of multiple sclerosis. J Neuroimmunol *144*, 68-79.

Madsen, L.S., Andersson, E.C., Jansson, L., krogsgaard, M., Andersen, C.B., Engberg, J., Strominger, J.L., Svejgaard, A., Hjorth, J.P., Holmdahl, R.*, et al.* (1999). A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor. Nat Genet *23*, 343-347.

Maeda, A., and Sobel, R.A. (1996). Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions. J Neuropathol Exp Neurol *55*, 300-309.

Magliozzi, R., Howell, O., Vora, A., Serafini, B., Nicholas, R., Puopolo, M., Reynolds, R., and Aloisi, F. (2007). Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain *130*, 1089-1104.

Magnus, T., Chan, A., Grauer, O., Toyka, K.V., and Gold, R. (2001). Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. J Immunol *167*, 5004-5010.

Manna, S.K., and Ramesh, G.T. (2005). Interleukin-8 induces nuclear transcription factor-kappaB through a TRAF6-dependent pathway. J Biol Chem *280*, 7010-7021.

Margueron, R., Trojer, P., and Reinberg, D. (2005). The key to development: interpreting the histone code? Curr Opin Genet Dev *15*, 163-176.

Marik, C., Felts, P.A., Bauer, J., Lassmann, H., and Smith, K.J. (2007). Lesion genesis in a subset of patients with multiple sclerosis: a role for innate immunity? Brain *130*, 2800-2815.

Marrie, R.A. (2004). Environmental risk factors in multiple sclerosis aetiology. The Lancet Neurology *3*, 709-718.

Marta, M., Andersson, A., Isaksson, M., Kampe, O., and Lobell, A. (2008). Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. Eur J Immunol *38*, 565-575.

Marta, M., Meier, U.C., and Lobell, A. (2009). Regulation of autoimmune encephalomyelitis by toll-like receptors. Autoimmun Rev *8*, 506-509.

Martin, C., and Zhang, Y. (2005). The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol *6*, 838-849.

Mastronardi, F.G., Noor, A., Wood, D.D., Paton, T., and Moscarello, M.A. (2007). Peptidyl argininedeiminase 2 CpG island in multiple sclerosis white matter is hypomethylated. J Neurosci Res *85*, 2006-2016.

McCoy, L., Tsunoda, I., and Fujinami, R.S. (2006). Multiple sclerosis and virus induced immune responses: autoimmunity can be primed by molecular mimicry and augmented by bystander activation. Autoimmunity *39*, 9-19.

McGuinness, M.C., Powers, J.M., Bias, W.B., Schmeckpeper, B.J., Segal, A.H., Gowda, V.C., Wesselingh, S.L., Berger, J., Griffin, D.E., and Smith, K.D. (1997). Human leukocyte antigens and cytokine expression in cerebral inflammatory demyelinative lesions of X-linked adrenoleukodystrophy and multiple sclerosis. J Neuroimmunol *75*, 174-182.

McManus, C., Berman, J.W., Brett, F.M., Staunton, H., Farrell, M., and Brosnan, C.F. (1998). MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. J Neuroimmunol *86*, 20-29.

McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J., and Sharp, P.A. (2002). Gene silencing using micro-RNA designed hairpins. RNA *8*, 842-850.

Medana, I., Martinic, M.A., Wekerle, H., and Neumann, H. (2001). Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. Am J Pathol *159*, 809-815.

Medzhitov, R., and Janeway, C.A., Jr. (1998). Innate immune recognition and control of adaptive immune responses. Semin Immunol *10*, 351-353.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway Jr, C.A. (1998). MyD88 Is an Adaptor Protein in the hToll/IL-1 Receptor Family Signaling Pathways. Molecular Cell *2*, 253-258.

Mei, I.A.F.v.d., Ponsonby, A.-L., Dwyer, T., Blizzard, L., Simmons, R., Taylor, B.V., Butzkueven, H., and Kilpatrick, T. (2003). Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. BMJ *327*, 316.

Mendel, I., Kerlero de Rosbo, N., and Ben-Nun, A. (1995). A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. Eur J Immunol *25*, 1951-1959.

Merrill, J.E., Ignarro, L.J., Sherman, M.P., Melinek, J., and Lane, T.E. (1993). Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. The Journal of Immunology *151*, 2132-2141.

Meurs, E., Chong, K., Galabru, J., Thomas, N.S.B., Kerr, I.M., Williams, B.R.G., and Hovanessian, A.G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell *62*, 379-390.

Miller, S.A., Mohn, S.E., and Weinmann, A.S. (2010). Jmjd3 and UTX Play a Demethylase-Independent Role in Chromatin Remodeling to Regulate T-Box Family Member-Dependent Gene Expression. Molecular Cell *40*, 594-605.

Min, I.M., Waterfall, J.J., Core, L.J., Munroe, R.J., Schimenti, J., and Lis, J.T. (2011). Regulating RNA polymerase pausing and transcription elongation in embryonic stem cells. Genes & Development *25*, 742-754.

Moran, L.B., Duke, D.C., and Graeber, M.B. (2007). The microglial gene regulatory network activated by interferon-gamma. Journal of Neuroimmunology *183*, 1-6.

Moran, L.B., Duke, D.C., Turkheimer, F.E., Banati, R.B., and Graeber, M.B. (2004). Towards a transcriptome definition of microglial cells. Neurogenetics *5*, 95-108.

Morris-Downes, M.M., Smith, P.A., Rundle, J.L., Piddlesden, S.J., Baker, D., Pham-Dinh, D., Heijmans, N., and Amor, S. (2002). Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. J Neuroimmunol *125*, 114-124.

Moscarello, M.A., Wood, D.D., Ackerley, C., and Boulias, C. (1994). Myelin in multiple sclerosis is developmentally immature. J Clin Invest *94*, 146-154.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol *136*, 2348-2357.

Mosser, D.M., and Edwards, J.P. (2008). Exploring the full spectrum of macrophage activation. Nat Rev Immunol *8*, 958-969.

Mott, R.T., Ait-Ghezala, G., Town, T., Mori, T., Vendrame, M., Zeng, J., Ehrhart, J., Mullan, M., and Tan, J. (2004). Neuronal expression of CD22: Novel mechanism for inhibiting microglial proinflammatory cytokine production. Glia *46*, 369-379.

Munger Kl, L.L.I.H.B.W.H.N.S.A.A. (2006). SErum 25-hydroxyvitamin d levels and risk of multiple sclerosis. JAMA *296*, 2832-2838.

Munoz, J.J., and Mackay, I.R. (1984). Production of experimental allergic encephalomyelitis with the aid of pertussigen in mouse strains considered genetically resistant. J Neuroimmunol *7*, 91-96.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science *272*, 263-267.

Narlikar, G.J., Fan, H.-Y., and Kingston, R.E. (2002). Cooperation between Complexes that Regulate Chromatin Structure and Transcription. Cell *108*, 475-487.

Natoli, G., Saccani, S., Bosisio, D., and Marazzi, I. (2005). Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. Nat Immunol *6*, 439-445.

Neumann, H., and Takahashi, K. (2007). Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis. Journal of Neuroimmunology *184*, 92-99.

Nightingale, K.P., O'Neill, L.P., and Turner, B.M. (2006). Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev *16*, 125-136.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science *308*, 1314-1318.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K.*, et al.* (2000). Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates with Down-Regulation of Surface Toll-Like Receptor 4 Expression. The Journal of Immunology *164*, 3476-3479.

Noseworthy, J.H., Lucchinetti, C., Rodriguez, M., and Weinshenker, B.G. (2000). Multiple sclerosis. N Engl J Med *343*, 938-952.

O'Keefe, G.M., Nguyen, V.T., and Benveniste, E.N. (2002). Regulation and Function of Class II Major Histocompatibility Complex, CD40, and B7 Expression in Macrophages and Microglia: Implications in Neurological Diseases. Journal of Neurovirology *8*, 496-512.

Oberdoerffer, P., Kanellopoulou, C., Heissmeyer, V., Paeper, C., Borowski, C., Aifantis, I., Rao, A., and Rajewsky, K. (2005). Efficiency of RNA Interference in the Mouse Hematopoietic System Varies between Cell Types and Developmental Stages. Molecular and Cellular Biology *25*, 3896-3905.

Ohashi, K., Burkart, V., Flohé, S., and Kolb, H. (2000). Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex. The Journal of Immunology *164*, 558-561.

Oksenberg, J.R., Baranzini, S.E., Sawcer, S., and Hauser, S.L. (2008). The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. Nat Rev Genet *9*, 516-526.

Oksenberg, J.R., Barcellos, L.F., Cree, B.A.C., Baranzini, S.E., Bugawan, T.L., Khan, O., Lincoln, R.R., Swerdlin, A., Mignot, E., Lin, L.*, et al.* (2004). Mapping Multiple Sclerosis Susceptibility to the HLA-DR Locus in African Americans. American journal of human genetics *74*, 160-167.

Okun, E., Griffioen, K.J., Lathia, J.D., Tang, S.C., Mattson, M.P., and Arumugam, T.V. (2009). Toll-like receptors in neurodegeneration. Brain Res Rev *59*, 278-292.

Olerup, O., and Hillert, J. (1991). HLA class II-associated genetic susceptibility in multiple sclerosis: A critical evaluation. Tissue Antigens *38*, 1-15.

Olson, J.K., Girvin, A.M., and Miller, S.D. (2001). Direct activation of innate and antigen-presenting functions of microglia following infection with Theiler's virus. J Virol *75*, 9780-9789.

Olson, J.K., and Miller, S.D. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol *173*, 3916-3924.

Olsson, T., Zhi, W.W., xF, jeberg, B., Kostulas, V., Jiang, Y.P., Anderson, G., Ekre, H.P., and Link, H. (1990). Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma. The Journal of Clinical Investigation *86*, 981-985.

Orton, S.M., Herrera, B.M., Yee, I.M., Valdar, W., Ramagopalan, S.V., Sadovnick, A.D., and Ebers, G.C. (2006). Sex ratio of multiple sclerosis in Canada: a longitudinal study. Lancet Neurol *5*, 932-936.

Ota, K., Matsui, M., Milford, E.L., Mackin, G.A., Weiner, H.L., and Hafler, D.A. (1990). T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature *346*, 183-187.

Özenci, V., Kouwenhoven, M., Huang, Y.M., Kivisäkk, P., and Link, H. (2000). Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-α)- and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-β) treatment. Clinical & Experimental Immunology *120*, 147-153.

Pace, J.L., Russell, S.W., Torres, B.A., Johnson, H.M., and Gray, P.W. (1983). Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. J Immunol *130*, 2011-2013.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev *16*, 948-958.

Pang, Y., Cai, Z., and Rhodes, P.G. (2003). Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. Developmental Brain Research *140*, 205-214.

Park, F., Ohashi, K., Chiu, W., Naldini, L., and Kay, M.A. (2000). Efficient lentiviral transduction of liver requires cell cycling in vivo. Nat Genet *24*, 49-52.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q.*, et al.* (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol *6*, 1133-1141.

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.-Y., Strassheim, D., Ishizaka, A., and Abraham, E. (2004). Involvement of Toll-like Receptors 2 and 4 in Cellular Activation by High Mobility Group Box 1 Protein. Journal of Biological Chemistry *279*, 7370-7377.

Pasini, D., Cloos, P.A.C., Walfridsson, J., Olsson, L., Bukowski, J.-P., Johansen, J.V., Bak, M., Tommerup, N., Rappsilber, J., and Helin, K. (2010). JARID2 regulates binding of the Polycomb repressive complex[thinsp]2 to target genes in ES cells. Nature *464*, 306-310.

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P.S., Bruck, W., Lucchinetti, C., and Lassmann, H. (2006). Remyelination is extensive in a subset of multiple sclerosis patients. Brain *129*, 3165-3172.

Pearson, V.L., Rothwell, N.J., and Toulmond, S. (1999). Excitotoxic brain damage in the rat induces interleukin-1beta protein in microglia and astrocytes: correlation with the progression of cell death. Glia *25*, 311-323.

Pedre, X., Mastronardi, F., Bruck, W., Lopez-Rodas, G., Kuhlmann, T., and Casaccia, P. (2011). Changed histone acetylation patterns in normal-appearing white matter and early multiple sclerosis lesions. J Neurosci *31*, 3435-3445.

Piccio, L., Buonsanti, C., Mariani, M., Cella, M., Gilfillan, S., Cross, A.H., Colonna, M., and Panina-Bordignon, P. (2007). Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis. European Journal of Immunology *37*, 1290-1301.

Pinteaux, E., Parker, L.C., Rothwell, N.J., and Luheshi, G.N. (2002). Expression of interleukin-1 receptors and their role in interleukin-1 actions in murine microglial cells. J Neurochem *83*, 754-763.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C.*, et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085-2088.

Ponomarev, E.D., Shriver, L.P., Maresz, K., and Dittel, B.N. (2005). Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. Journal of Neuroscience Research *81*, 374-389.

Powell, M.B., Mitchell, D., Lederman, J., Buckmeier, J., Zamvil, S.S., Graham, M., Ruddle, N.H., and Steinman, L. (1990). Lymphotoxin and tumor necrosis factor-alpha production by myelin basic protein-specific T cell clones correlates with encephalitogenicity. Int Immunol *2*, 539-544.

Prat, A., Biernacki, K., Lavoie, J.F., Poirier, J., Duquette, P., and Antel, J.P. (2002). Migration of multiple sclerosis lymphocytes through brain endothelium. Arch Neurol *59*, 391-397.

Prinz, M., Garbe, F., Schmidt, H., Mildner, A., Gutcher, I., Wolter, K., Piesche, M., Schroers, R., Weiss, E., Kirschning, C.J.*, et al.* (2006). Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. J Clin Invest *116*, 456-464.

Qiu, H., Hu, C., Yoon, S., Natarajan, K., Swanson, M.J., and Hinnebusch, A.G. (2004). An Array of Coactivators Is Required for Optimal Recruitment of TATA Binding Protein and RNA Polymerase II by Promoter-Bound Gcn4p. Molecular and Cellular Biology *24*, 4104-4117.

Raivich, G., and Banati, R. (2004). Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. Brain Research Reviews *46*, 261-281.

Ramagopalan, S.V., Dobson, R., Meier, U.C., and Giovannoni, G. (2010). Multiple sclerosis: risk factors, prodromes, and potential causal pathways. The Lancet Neurology *9*, 727-739.

Ransohoff, R.M., and Cardona, A.E. (2010). The myeloid cells of the central nervous system parenchyma. Nature *468*, 253-262.

Ransohoff, R.M., and Perry, V.H. (2009). Microglial physiology: unique stimuli, specialized responses. Annu Rev Immunol *27*, 119-145.

Rasmussen, S., Wang, Y., Kivisakk, P., Bronson, R.T., Meyer, M., Imitola, J., and Khoury, S.J. (2007). Persistent activation of microglia is associated with neuronal dysfunction of callosal projecting pathways and multiple sclerosis-like lesions in relapsing--remitting experimental autoimmune encephalomyelitis. Brain *130*, 2816-2829.

Ringrose, L., and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development *134*, 223-232.

Rivers, T.M., Sprunt, D.H., and Berry, G.P. (1933). Observations on Attempts to Produce Acute Disseminated Encephalomyelitis in Monkeys. J Exp Med *58*, 39-53.

Robertson, N.P., Fraser, M., Deans, J., Clayton, D., Walker, N., and Compston, D.A.S. (1996). Age–adjusted recurrence risks for relatives of patients with multiple sclerosis. Brain *119*, 449-455.

Rodriguez, M., Leibowitz, J.L., and Lampert, P.W. (1983). Persistent infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. Ann Neurol *13*, 426-433.

Roeder, R.G. (2005). Transcriptional regulation and the role of diverse coactivators in animal cells. FEBS letters *579*, 909-915.

Root, D.E., Hacohen, N., Hahn, W.C., Lander, E.S., and Sabatini, D.M. (2006). Genome-scale loss-of-function screening with a lentiviral RNAi library. Nat Meth *3*, 715-719.

Sadovnick, A.D., Armstrong, H., Rice, G.P.A., Bulman, D., Hashimoto, L., Party, D.W., Hashimoto, S.A., Warren, S., Hader, W., Murrary, T.J.*, et al.* (1993). A population-based study of multiple sclerosis in twins: Update. Annals of Neurology *33*, 281-285.

Sadovnick, A.D., Baird, P.A., Ward, R.H., Optiz, J.M., and Reynolds, J.F. (1988). Multiple sclerosis. Updated risks for relatives. American Journal of Medical Genetics *29*, 533-541.

Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Tanaka, Y., Kumagai, Y., Miyake, T., Matsushita, K., Okazaki, T., Saitoh, T.*, et al.* (2010). The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol.

Saunders, A., Core, L.J., and Lis, J.T. (2006). Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol *7*, 557-567.

Saura, J., Tusell, J.M., and Serratosa, J. (2003). High-yield isolation of murine microglia by mild trypsinization. Glia *44*, 183-189.

Scolding, N., Franklin, R., Stevens, S., Heldin, C.H., Compston, A., and Newcombe, J. (1998). Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. Brain *121 (Pt 12)*, 2221-2228.

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., and Plank, C. (2002). Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther *9*, 102-109.

Schluesener, H.J., and Wekerle, H. (1985). Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. J Immunol *135*, 3128-3133.

Schmid, C.D., Melchior, B., Masek, K., Puntambekar, S.S., Danielson, P.E., Lo, D.D., Gregor Sutcliffe, J., and Carson, M.J. (2009). Differential gene expression in LPS/IFNγ activated microglia and macrophages: in vitro versus in vivo. Journal of Neurochemistry *109*, 117-125.

Schroder, J.M., Mrowietz, U., Morita, E., and Christophers, E. (1987). Purification and partial biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. J Immunol *139*, 3474-3483.

Schuitemaker, H., Kootstra, N.A., Fouchier, R.A., Hooibrink, B., and Miedema, F. (1994). Productive HIV-1 infection of macrophages restricted to the cell fraction with proliferative capacity. EMBO J *13*, 5929-5936.

Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet *8*, 9-22.

Schwentker, F.F., and Rivers, T.M. (1934). The Antibody Response of Rabbits to Injections of Emulsions and Extracts of Homologous Brain. J Exp Med *60*, 559-574.

Sedgwick, J.D., Schwender, S., Imrich, H., Dörries, R., Butcher, G.W., and ter Meulen, V. (1991). Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proceedings of the National Academy of Sciences *88*, 7438-7442.

Segal, B.M., Dwyer, B.K., and Shevach, E.M. (1998). An Interleukin (IL)-10/IL-12 Immunoregulatory Circuit Controls Susceptibility to Autoimmune Disease. The Journal of Experimental Medicine *187*, 537-546.

Sen, G.L., Webster, D.E., Barragan, D.I., Chang, H.Y., and Khavari, P.A. (2008). Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. Genes Dev *22*, 1865-1870.

Sen, R., and Smale, S.T. (2010). Selectivity of the NF-{kappa}B response. Cold Spring Harb Perspect Biol *2*, a000257.

Shen, S., Li, J., and Casaccia-Bonnefil, P. (2005). Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. J Cell Biol *169*, 577-589.

Shen, S., Sandoval, J., Swiss, V.A., Li, J., Dupree, J., Franklin, R.J.M., and Casaccia-Bonnefil, P. (2008). Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency. Nat Neurosci *11*, 1024-1034.

Shi, Y., Feng, Y., Kang, J., Liu, C., Li, Z., Li, D., Cao, W., Qiu, J., Guo, Z., Bi, E.*, et al.* (2007). Critical regulation of CD4+ T cell survival and autoimmunity by [beta]-arrestin 1. Nat Immunol *8*, 817-824.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R.*, et al.* (2005). Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet *37*, 1281-1288.

Smiley, S.T., King, J.A., and Hancock, W.W. (2001). Fibrinogen Stimulates Macrophage Chemokine Secretion Through Toll-Like Receptor 4. The Journal of Immunology *167*, 2887-2894.

Smith, E.R., Lee, M.G., Winter, B., Droz, N.M., Eissenberg, J.C., Shiekhattar, R., and Shilatifard, A. (2008). Drosophila UTX is a histone H3 Lys27 demethylase that colocalizes with the elongating form of RNA polymerase II. Mol Cell Biol *28*, 1041-1046.

Smith, K.J., Blakemore, W.F., and McDonald, W.I. (1981). The restoration of conduction by central remyelination. Brain *104*, 383-404.

Smith, K.J., Kapoor, R., Hall, S.M., and Davies, M. (2001). Electrically active axons degenerate when exposed to nitric oxide. Ann Neurol *49*, 470-476.

Soldan, S.S., Berti, R., Salem, N., Secchiero, P., Flamand, L., Calabresi, P.A., Brennan, M.B., Maloni, H.W., McFarland, H.F., Lin, H.-C*., et al.* (1997). Association of human herpes virus 6 (HHV-6) with multiple sclerosis: Increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. Nat Med *3*, 1394-1397.

Sospedra, M., and Martin, R. (2005). Immunology of multiple sclerosis. Annu Rev Immunol *23*, 683-747.

Spannhoff, A., Hauser, A.T., Heinke, R., Sippl, W., and Jung, M. (2009). The emerging therapeutic potential of histone methyltransferase and demethylase inhibitors. ChemMedChem *4*, 1568-1582.

Spina, C.A., Guatelli, J.C., and Richman, D.D. (1995). Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J Virol *69*, 2977-2988.

Sriram, S. (2011). Role of glial cells in innate immunity and their role in CNS demyelination. Journal of Neuroimmunology *239*, 13-20.

Steinman, L., Martin, R., Bernard, C., Conlon, P., and Oksenberg, J.R. (2002). Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. Annu Rev Neurosci *25*, 491-505.

Stenger, S., and Modlin, R.L. (2002). Control of Mycobacterium tuberculosis through mammalian Toll-like receptors. Current Opinion in Immunology *14*, 452-457.

Stevens, S.L., Bao, J., Hollis, J., Lessov, N.S., Clark, W.M., and Stenzel-Poore, M.P. (2002). The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. Brain Res *932*, 110-119.

Storch, M.K., Piddlesden, S., Haltia, M., Iivanainen, M., Morgan, P., and Lassmann, H. (1998). Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. Ann Neurol *43*, 465-471.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Streit, W.J., Graeber, M.B., and Kreutzberg, G.W. (1989). Expression of Ia antigen on perivascular and microglial cells after sublethal and lethal motor neuron injury. Experimental Neurology *105*, 115-126.

Sugawara, H., Iwamoto, K., Bundo, M., Ueda, J., Ishigooka, J., and Kato, T. (2011). Comprehensive DNA methylation analysis of human peripheral blood leukocytes and lymphoblastoid cell lines. Epigenetics *6*, 508-515.

Sun, D., Whitaker, J.N., Huang, Z., Liu, D., Coleclough, C., Wekerle, H., and Raine, C.S. (2001). Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. J Immunol *166*, 7579-7587.

Surani, M.A., Hayashi, K., and Hajkova, P. (2007). Genetic and Epigenetic Regulators of Pluripotency. Cell *128*, 747-762.

T.I.M.S.G.C.T.W.T.C.C.C (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature *476*, 214-219.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Taylor, K.R., Trowbridge, J.M., Rudisill, J.A., Termeer, C.C., Simon, J.C., and Gallo, R.L. (2004). Hyaluronan Fragments Stimulate Endothelial Recognition of Injury through TLR4. Journal of Biological Chemistry *279*, 17079-17084.

Thacker, E.L., Mirzaei, F., and Ascherio, A. (2006). Infectious mononucleosis and risk for multiple sclerosis: A meta-analysis. Annals of Neurology *59*, 499-503.

Thiesen, S., Kübart, S., Ropers, H.H., and Nothwang, H.G. (2000). Isolation of Two Novel Human RhoGEFs, ARHGEF3 and ARHGEF4, in 3p13-21 and 2q22. Biochemical and Biophysical Research Communications *273*, 364-369.

Tiscornia, G., Singer, O., and Verma, I.M. (2006a). Design and cloning of lentiviral vectors expressing small interfering RNAs. Nat Protoc *1*, 234-240.

Tiscornia, G., Singer, O., and Verma, I.M. (2006b). Production and purification of lentiviral vectors. Nat Protoc *1*, 241-245.

Touil, T., Fitzgerald, D., Zhang, G.X., Rostami, A., and Gran, B. (2006). Cutting Edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta. J Immunol *177*, 7505-7509.

Town, T., Jeng, D., Alexopoulou, L., Tan, J., and Flavell, R.A. (2006). Microglia Recognize Double-Stranded RNA via TLR3. The Journal of Immunology *176*, 3804-3812.

Trapp, B.D., Wujek, J.R., Criste, G.A., Jalabi, W., Yin, X., Kidd, G.J., Stohlman, S., and Ransohoff, R. (2007). Evidence for synaptic stripping by cortical microglia. Glia *55*, 360-368.

Trono, D. (2002). Lentiviral vectors (Berlin ; London, Springer).

Trotter, J.L., Clark, H.B., Collins, K.G., Wegeschiede, C.L., and Scarpellini, J.D. (1987). Myelin proteolipid protein induces demyelinating disease in mice. J Neurol Sci *79*, 173-188.

Tsuji, D., Kuroki, A., Ishibashi, Y., Itakura, T., and Itoh, K. (2005). Metabolic correction in microglia derived from Sandhoff disease model mice. J Neurochem *94*, 1631-1638.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

Tsunoda, I., and Fujinami, R.S. (1996). Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. J Neuropathol Exp Neurol *55*, 673-686.

Tuohy, V.K., and Thomas, D.M. (1995). Sequence 104–117 of myelin proteolipid protein is a cryptic encephalitogenic T cell determinant for SJL/J mice. Journal of Neuroimmunology *56*, 161-170.

Turner, B.M. (2007). Defining an epigenetic code. Nat Cell Biol *9*, 2-6.

Tzartos, J.S., Friese, M.A., Craner, M.J., Palace, J., Newcombe, J., Esiri, M.M., and Fugger, L. (2008). Interleukin-17 Production in Central Nervous System-Infiltrating T Cells and Glial Cells Is Associated with Active Disease in Multiple Sclerosis. The American Journal of Pathology *172*, 146-155.

Unutmaz, D., KewalRamani, V.N., Marmon, S., and Littman, D.R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J Exp Med *189*, 1735-1746.

van Furth, R., and Cohn, Z.A. (1968). THE ORIGIN AND KINETICS OF MONONUCLEAR PHAGOCYTES. The Journal of Experimental Medicine *128*, 415-435.

Vergo, S., Craner, M.J., Etzensperger, R., Attfield, K., Friese, M.A., Newcombe, J., Esiri, M., and Fugger, L. (2011). Acid-sensing ion channel 1 is involved in both axonal injury and demyelination in multiple sclerosis and its animal model. Brain *134*, 571-584.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of Functional Suppression by CD4+CD25+ Regulatory T Cells in Patients with Multiple Sclerosis. The Journal of Experimental Medicine *199*, 971-979.

Vollmer, J., Tluk, S., Schmitz, C., Hamm, S., Jurk, M., Forsbach, A., Akira, S., Kelly, K.M., Reeves, W.H., Bauer, S.*, et al.* (2005). Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. The Journal of Experimental Medicine *202*, 1575-1585.

Waldner, H., Collins, M., and Kuchroo, V.K. (2004). Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. The Journal of Clinical Investigation *113*, 990-997.

Waldner, H., Whitters, M.J., Sobel, R.A., Collins, M., and Kuchroo, V.K. (2000). Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic for the myelin proteolipid protein-specific T cell receptor. Proc Natl Acad Sci U S A *97*, 3412-3417.

Wallin, M.T., Page, W.F., and Kurtzke, J.F. (2004). Multiple sclerosis in US veterans of the Vietnam era and later military service: Race, sex, and geography. Annals of Neurology *55*, 65-71.

Waxman, S.G., and Brill, M.H. (1978). Conduction through demyelinated plaques in multiple sclerosis: computer simulations of facilitation by short internodes. J Neurol Neurosurg Psychiatry *41*, 408-416.

Weake, V.M., and Workman, J.L. (2010). Inducible gene expression: diverse regulatory mechanisms. Nat Rev Genet *11*, 426-437.

Wechsler, A.S., Gordon, M.C., Dendorfer, U., and LeClair, K.P. (1994). Induction of IL-8 expression in T cells uses the CD28 costimulatory pathway. The Journal of Immunology *153*, 2515-2523.

Weinshenker, B.G., Bass, B., Rice, G.P., Noseworthy, J., Carriere, W., Baskerville, J., and Ebers, G.C. (1989). The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. Brain *112 (Pt 1)*, 133-146.

Wekerle, H. (1993). Experimental autoimmune encephalomyelitis as a model of immune-mediated CNS disease. Curr Opin Neurobiol *3*, 779-784.

Wilkins, A., Majed, H., Layfield, R., Compston, A., and Chandran, S. (2003). Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. J Neurosci *23*, 4967-4974.

Wilson, C.B., Rowell, E., and Sekimata, M. (2009). Epigenetic control of T-helper-cell differentiation. Nat Rev Immunol *9*, 91-105.

Windhagen, A., Newcombe, J., Dangond, F., Strand, C., Woodroofe, M.N., Cuzner, M.L., and Hafler, D.A. (1995). Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. The Journal of Experimental Medicine *182*, 1985-1996.

Wolf, A., Kabat, E.A., and Bezer, A.E. (1947). The pathology of acute disseminated encephalomyelitis produced experimentally in the rhesus monkey and its resemblance to human demyelinating disease. J Neuropathol Exp Neurol *6*, 333-357.

Wolfson, C., and Talbot, P. (2002). Bacterial infection as a cause of multiple sclerosis. Lancet *360*, 352-353.

Wolswijk, G. (1998). Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. J Neurosci *18*, 601-609.

xF, rensen, T.L., Tani, M., Jensen, J., Pierce, V., Lucchinetti, C., Folcik, V.A., Qin, S., Rottman, J., Sellebjerg, F*., et al.* (1999). Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. The Journal of Clinical Investigation *103*, 807-815.

Xiang, Y., Zhu, Z., Han, G., Lin, H., Xu, L., and Chen, C.D. (2007). JMJD3 is a histone H3K27 demethylase. Cell Res *17*, 850-857.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K.*, et al.* (2003). Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. Science *301*, 640-643.

Yeo, T.W., De Jager, P.L., Gregory, S.G., Barcellos, L.F., Walton, A., Goris, A., Fenoglio, C., Ban, M., Taylor, C.J., Goodman, R.S.*, et al.* (2007). A second major histocompatibility complex susceptibility locus for multiple sclerosis. Annals of Neurology *61*, 228-236.

Youl, B.D., Turano, G., Miller, D.H., Towell, A.D., MacManus, D.G., Moore, S.G., Jones, S.J., Barrett, G., Kendall, B.E., Moseley, I.F.*, et al.* (1991). The pathophysiology of acute optic neuritis. An association of gadolinium leakage with clinical and electrophysiological deficits. Brain *114 (Pt 6)*, 2437-2450.

Young, M.D., Willson, T.A., Wakefield, M.J., Trounson, E., Hilton, D.J., Blewitt, M.E., Oshlack, A., and Majewski, I.J. (2011). ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. Nucleic Acids Research *39*, 7415-7427.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., and Chen, I.S. (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell *61*, 213-222.

Zamanian-Daryoush, M., Mogensen, T.H., DiDonato, J.A., and Williams, B.R.G. (2000). NF-κB Activation by Double-Stranded-RNA-Activated Protein Kinase (PKR) Is Mediated through NF-κB-Inducing Kinase and IκB Kinase. Molecular and Cellular Biology *20*, 1278-1290.

Zamvil, S.S., Mitchell, D.J., Powell, M.B., Sakai, K., Rothbard, J.B., and Steinman, L. (1988). Multiple discrete encephalitogenic epitopes of the autoantigen myelin basic protein include a determinant for I-E class II-restricted T cells. J Exp Med *168*, 1181-1186.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., and Kagan, J.C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell *147*, 868-880.

Zeis, T., Graumann, U., Reynolds, R., and Schaeren-Wiemers, N. (2008). Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. Brain *131*, 288-303.

Zhang, Q., long, H., Liao, J., Zhao, M., Liang, G., Wu, X., Zhang, P., Ding, S., Luo, S., and Lu, Q. (2011). Inhibited expression of hematopoietic progenitor kinase 1 associated with loss of jumonji domain containing 3 promoter binding contributes to autoimmunity in systemic lupus erythematosus. Journal of Autoimmunity *37*, 180-189.

Zhang, Z., and Schluesener, H.J. (2006). Mammalian toll-like receptors: from endogenous ligands to tissue regeneration. Cell Mol Life Sci *63*, 2901-2907.

Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K.A., Reimann, K.A., Reinhart, T.A., Rogan, M., Cavert, W., Miller, C.J.*, et al.* (1999). Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science *286*, 1353-1357.