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## The role of differently activated macrophages in axonal damage during multiple sclerosis

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# CHAPTER

# 1

## GENERAL INTRODUCTION

Adapted from:  
Macrophages and axonal damage and repair in multiple sclerosis  
Vereyken E.J.F., Dijkstra C.D., Teunissen C.E.

## MULTIPLE SCLEROSIS

### *Clinical symptoms and diagnosis in vitro*

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The prevalence of MS is approximately 2 million people worldwide, with an incidence of about 1:1000 in Europe and Northern America, and women are affected more often compared to men, at a ratio of approximately 2:1. It is the most common cause of neurological disability among young adults with an onset generally between 20 and 40 years of age<sup>1</sup>. MS is characterized by multiple sclerotic lesions affecting areas such as the cerebellum, cerebrum (periventricular white matter), optic nerve, brainstem and spinal cord<sup>2</sup>. The clinical symptoms of MS are very heterogeneous depending on lesion location, size and number. The symptoms include motor function disturbances, such as muscle weakness, tremor and paralysis, and progressive sensory malfunction, for instance impaired vision. The main criterium for the diagnosis of MS is the occurrence of two (or more) independent episodes of clinical symptoms consistent with focal demyelination separated in space (part of the CNS) and time (more than one occasion)<sup>3</sup>. Magnetic resonance imaging (MRI) techniques have become very important for the verification of the diagnosis<sup>4-6</sup>, for the detection of the number and size of the lesions and to differentiate between ongoing inflammation and blood-brain-barrier leakage<sup>7-10</sup>. In the cerebrospinal fluid (CSF) of MS patients abnormalities can be detected, such as the presence of elevated immunoglobulin G (IgG) levels and the identification of two or more unique oligoclonal bands<sup>11</sup>. In approximately 90% of MS patients oligoclonal immunoglobulin bands are detected<sup>12</sup>. These oligoclonal bands are also detected in other neurological diseases, although not as consistently or as persistently as in MS. In patients with clinically isolated syndrome the presence of oligoclonal bands is predictive for the progression to clinically definite MS<sup>13</sup>. In MS patients the absence of oligoclonal bands has been associated with a benign disease course, while high levels of oligoclonal bands have been correlated with a severe disease, suggesting the oligoclonal bands may be clinically relevant<sup>11</sup>.

Four major subtypes of MS with different progression and relapse characteristics have been recognized: the relapsing-remitting (RR-MS), the secondary-progressive (SP-MS), primary progressive (PP-MS) and progressive-relapsing (PR-MS) subtype<sup>14</sup>. Approximately 70% of the cases start with the RR subtype, which is characterized by clinical attacks that are followed by a clinically silent period with almost complete recovery. After a period of 15 to 20 years most cases of the RR-MS subtype develop progressive neurological deterioration without apparent remission, the SP-MS subtype. About 15-20% of MS patients show a progressive disease course without relapses and remissions from the beginning, the PP-MS subtype. Finally, less than 5% suffer from PR-MS, characterized by progressive neurological impairment with occasional relapses.

### *Pathology*

The major neuropathological hallmarks of MS are multiple focal inflammatory

demyelinating lesions spread throughout the CNS. These lesions are associated with perivascular infiltrates containing macrophages and lymphocytes. Other features of MS plaques are axonal damage and loss, oligodendrocyte death and astrogliosis<sup>15</sup>, which is hypertrophy and an abnormal increase in the number of astrocytes. Lesions are classified based on the degree of myelin loss, the presence of inflammatory cells and HLA-DR expression on leukocytes and microglial cells. Four different stages in MS lesions have been identified: (p)reactive, active, chronic active and chronic inactive lesions<sup>16;17</sup>.

In (p)reactive lesions no demyelination is apparent. Clusters of activated microglia can be observed with increased expression of HLA-DR expression and occasionally perivascular leukocyte infiltrations can be seen.

Active lesions are characterized by areas of demyelination containing macrophages, activated microglia and activated hypertrophic astrocytes. Activated astrocytes fill up the lesion area and form a gliotic scar. T-cells and some B-cells can be found, mostly in the perivascular space. The macrophages and microglia in the lesions contain myelin degradation products, such as myelin proteins and lipids, giving them a foamy appearance. The presence of myelin proteins in these macrophages reflect ongoing demyelination taking place<sup>18;19</sup>. Oligodendrocyte death occurs in these lesions, often via apoptotic mechanisms<sup>20;21</sup>. This apoptosis of oligodendrocytes may be a disease initiating event, since it precedes leukocyte infiltration<sup>22;23</sup>.

Chronic active lesions are defined by a hypocellular demyelinated centre surrounded by a hypercellular rim with high numbers of foamy macrophages and reactive astrocytes. In these lesions oligodendrocyte numbers are reduced and lymphocytes are present in perivascular spaces.

In chronic inactive lesions almost no cellular infiltrates are present. They are hypocellular, demyelinated and contain widened extracellular spaces and gliotic scar tissue. In the CNS parenchyma and perivascular spaces relatively small numbers of macrophages and lymphocytes still remain. No myelin proteins can be detected in the macrophages.

Next to demyelination, remyelination also occurs<sup>24</sup>. Remyelination can be restricted to the lesion edge, but can also extend throughout the lesions which are then called shadow-plaques<sup>25-29</sup>. Oligodendrocyte precursor cells, after maturation into mature oligodendrocytes, generate thin myelin sheaths and could therefore contribute to recovery in MS patients. Remyelination in MS is limited. The cause of remyelination failure in MS is unknown, but several mechanisms have been proposed, such as restricted oligodendrocyte precursor cell migration, maturation and a growth inhibitory environment<sup>24;30-32</sup>.

## AXONAL DAMAGE IN MS

Historically, MS has been viewed as a primary demyelinating disease with relative axonal sparing, although early papers did describe axonal damage and loss<sup>33</sup>. However, the view that axonal damage is important in MS pathology is now being widely accepted. The earliest studies on MS pathology already described

axonal damage and loss <sup>15;16;34</sup>, although, this fact was obscured by the much more evident demyelination. Demyelination has long been considered the main cause of disability in MS. However, more recent reports suggest that axonal injury is the main correlate of irreversible clinical disability in MS patients <sup>35;36</sup> and experimental autoimmune encephalomyelitis (EAE), an animal model for MS <sup>37</sup>. Early axonal damage may be either compensated for and/or repaired, but the continuous progression of axonal loss could ultimately lead to irreversible clinical dysfunction. A current hypothesis poses that the transition from the RR to the SP subtype takes place once the loss of a critical number of axons is exceeded <sup>33</sup>.

An indication that axonal damage might be important in MS pathology was that axonal transections are common in MS lesions, even in the periplaque white matter <sup>38;39</sup>. It was most extensive in areas of active demyelination and inflammation <sup>40</sup>. Even in the earliest stages of the disease widespread axonal damage was observed <sup>41</sup>. In chronic MS patients, axonal density was significantly decreased, in both normal appearing white matter (NAWM) and lesions in the cervical spinal cord, compared to controls <sup>39;42</sup>. The decrease in axonal density was more extensive inside most lesions compared to the adjacent NAWM <sup>33</sup>. A marker for axonal injury is the amyloid precursor protein (APP) <sup>43;44</sup>. APP, a transmembrane protein that has been suggested to be involved in Notch signaling <sup>45</sup>, is transported axonally by means of the fast anterograde component <sup>46</sup>. During acute injury anterograde axonal transport is interrupted causing APP to accumulate. In lesions with active demyelination APP accumulation was found in so-called “bulbs”, suggesting that axonal damage is a feature of early pathology and possibly associated with inflammation <sup>47</sup>. In inactive lesions significant, though low-level axonal damage was observed associated with residual inflammation <sup>48</sup>.

Another marker for axonal damage is non-phosphorylated neurofilament (NP-NF) <sup>49</sup>. Caliber changes, axonal transections and terminal ovoids have been observed in MS lesions using this method. Discontinuous NP-NF staining suggests that axons undergo Wallerian degeneration, degeneration distal from the sites of transection in MS patients <sup>49;50</sup>. Also, in EAE alterations in the distribution of dendritic and synaptic proteins have been described, suggesting a loss of neuronal contacts in this model <sup>51</sup>. Finally, redistribution of sodium channels and sodium/calcium exchanger NCX on demyelinated axons was shown to associate with APP accumulation providing another marker for axonal damage <sup>52;53</sup>.

Magnetic resonance imaging (MRI) and spectroscopy (MRS) have been used to visualize neuronal damage in living patients. MRI parameters in the brain of RR- and SP-MS patients were found to correlate with Expanded Disability Status Scale (EDSS) scores, while in PP-MS spinal cord abnormalities, spinal cord cross section and the number of segments showing diffuse abnormalities, correlated better with clinical symptoms <sup>54</sup>. Next to specific lesions, brain atrophy was found to occur in MS patients <sup>55;56</sup>. Brain atrophy was found to be a better predictor than number of inflammatory lesions for disability in MS patients <sup>57;58</sup>. The findings of cerebellar cortical atrophy in EAE mice parallel findings in humans <sup>59</sup>. These findings suggest that brain atrophy leads to functional impairment.

A more specific marker for axonal damage in MS is N-acetylaspartate (NAA),

which can be detected *in vivo* in the brain using MRS <sup>35;60</sup>. NAA, a mitochondrial amino acid, is primarily localized in neurons and neuronal processes, which means that a reduction in NAA signal reflects axonal injury and/or neuronal loss <sup>61</sup>. In both RR-MS and SP-MS patient groups a decrease in NAA:Creatinine ratio in both NAWM and lesions was observed compared to controls, with the decrease in lesion areas being higher compared to NAWM. The NAA:Creatinine ratio was significantly lower in the NAWM of SP-MS compared to RR-MS patients <sup>62;63</sup>. A strong indication that NAA levels measured by MRS might really reflect neuroaxonal integrity was the fact that a relationship was observed between the decrease in NAA levels measured by MRS in MS lesions and the decrease in axonal density in corresponding biopsy specimens <sup>38;64</sup>. A correlation was found between the reduction in NAA levels, motor conduction times and functional disability in 12 MS patients <sup>65</sup>. Furthermore, in RR-MS patients clinical disability as observed with EDSS correlated with brain NAA:Creatinine ratio <sup>62;66</sup>. The SP-MS patients did not show this correlation with EDSS, which might be due to lower disease activity and the small number of patients.

Changes in cerebrospinal fluid (CSF) concentrations of axon specific markers also point to a role of axonal damage in MS pathology and progression. In CSF of MS patients the NAA concentration was found to correlate with EDSS, a lower brain volume and a higher lesion load <sup>67</sup>. In the CSF of SP-MS patients decreased levels of NAA and increased levels of neurofilament heavy (NF H) were observed. Neurofilament light (NF L) levels were increased already in clinically isolated syndrome (CIS) patients, especially with those who converted to clinically definite MS, and correlated with the number of gadolinium enhancing lesions and relapses, suggesting that this marker has prognostic value <sup>68</sup>. The autoantibody index for the NF L chain has been found to correlate with atrophy <sup>69</sup>. Another biomarker for axonal damage in the CSF is tau.

All these data confirm the importance of axonal damage in MS pathology, which can already be observed early in the disease course. The mechanisms causing this damage are largely unknown. Several mechanisms have been proposed. First, demyelination has been may be involved in axonal damage. Myelin forms a physical barrier that protects axons from damaging agents. Furthermore myelin and oligodendrocytes seem to generate survival signals. In mice that do not form proper myelin, due to deficiency of myelin associated glycoprotein (MAG), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) or proteolipid protein (PLP), axons were found to show chronic atrophy and signs of degeneration <sup>70-74</sup>. Demyelination also leads to redistribution of sodium channels, in order to maintain proper signal propagation. This redistribution leads to enhanced energy metabolism and increased intracellular calcium concentrations, causing toxicity <sup>75</sup>. In MS lesions axons and astrocytes showed increased activity of complex IV and numbers of mitochondria, associated with an upregulation of the mitochondrial heat shock protein 70, indicative of oxidative stress in the mitochondria <sup>76</sup>. Furthermore, mitochondrial dysfunction due to oxidative stress, mediated by reactive oxygen species (ROS) produced by mitochondria themselves, has been observed early in EAE lesions, suggesting another mechanism for neuronal degeneration <sup>77</sup>. Another

hypothesis is that infiltrating macrophages might play a crucial role in axonal damage.

### **MACROPHAGES**

Macrophages (meaning “big eaters”) are phagocytic cells that play a vital role in innate immunity, the first line of defense against pathogens. Cells of the innate immune system such as macrophages are able, to some extent, to discriminate between “self” and “non-self” antigens (reviewed by Janeway <sup>78;79</sup>). Via a limited number of germline-encoded pattern recognition receptors, macrophages recognize highly conserved structures from bacteria, viruses and fungi. Several different families of pattern recognition receptors have been identified, for example macrophage scavenger receptors and Toll-like receptor family <sup>80;81</sup>. After recognition, the binding of the receptor with its ligand on the pathogen, macrophages usually engulf the pathogen, a process called phagocytosis. This process results in the containment of microbes in the phagosome, which fuses with lysosomal vesicles containing a multitude of microbicidal products. Both oxygen-dependent, called the respiratory burst, and oxygen-independent microbicidal mechanisms exist. The respiratory burst uses an enzymatic complex called nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase. Upon stimulation active NADPH oxidase forms. This active complex transfers two electrons from NADPH to two molecules of oxygen to form superoxide anion <sup>82;83</sup>. From this superoxide anion other ROS, such as hydrogen peroxide, are formed <sup>83</sup>. Oxygen-independent microbicidal mechanisms include acidification of the phagolysosome, nutrient depletion and antimicrobial proteins or peptides.

Macrophages differentiate from circulating monocytes. Once a monocyte migrates into a specific tissue, during steady-state or inflammation, they develop into macrophages. Macrophages are present in virtually all tissues and usually specialize according to the tissue they are in, for instance osteoclasts (in bone), Kupffer cells (in liver) and microglia (in the CNS) <sup>84</sup>.

Next to their role in innate immunity they have an important function in tissue homeostasis, since they are crucial for the clearance of apoptotic cells and the remodeling and repair of tissues after inflammation <sup>85;86</sup>. Phagocytosis of apoptotic cells does not induce the expression of inflammatory mediators in unstimulated macrophages <sup>87</sup>.

During an infection macrophages also clear cellular debris of necrotic cells that contain endogenous danger signals, such as heat-shock proteins and nuclear proteins <sup>88</sup>. The detection of these danger signals alters the physiology of the macrophages, including expression of cell surface proteins, cytokines and pro-inflammatory mediators, increasing immune function of macrophages. However, macrophages can respond to many signals in the microenvironment of tissues and not all increase immune function.

#### *Subtypes of macrophages*

Macrophages are highly plastic cells able to respond to a variety of

environmental cues changing their phenotype and physiology in response to these signals, resulting in different subtypes of macrophages. These different subtypes of macrophages have different functions in the immune response, homeostasis and tissue repair <sup>89-91</sup>.

Based on activation pathways several subtypes of macrophages have been described <sup>92,93</sup>. The two most studied subtypes are: 1) the classically activated macrophages (CA, also called M1), induced by interferon-gamma (IFN- $\gamma$ ) and lipopolysaccharide (LPS); 2) the alternatively activated macrophages (AA, also called M2), stimulated by IL-4 and/or glucocorticoids. In 1992 Stein et al. introduced the concept of alternatively activated macrophages <sup>94</sup>. In contrast to the classically activated macrophages, macrophages stimulated with interleukin-4 (IL-4) increased the expression of mannose receptor (MR). Another study showed that Th1 cytokines (e.g. IFN- $\gamma$ ) and Th2 cytokines (e.g. IL-4) induced two distinct functional states. Exposure of macrophages to Th2 cytokines led to an upregulation of certain phagocytic receptors and arginase, which reduced ability to kill intracellular pathogens, while Th1 cytokines led to induction of inducible nitric oxide synthase (iNOS) in macrophages <sup>95</sup>.

CA macrophages are cytotoxic and secrete high amounts of oxygen and nitrogen radicals in order to kill pathogens <sup>96</sup>. CA macrophages also produce pro-inflammatory cytokines <sup>97</sup>. In mice, CA macrophages are characterized by their production of nitric oxide (NO) <sup>92,98,99</sup>. Human macrophages derived from circulating monocytes do not generally produce NO <sup>90</sup>, therefore other markers should be used to discriminate between CA and AA macrophages, such as MR, E-cadherin <sup>100</sup>, CD40 <sup>101</sup> and Fc-gamma receptor I (Fc $\gamma$ RI). Markers for the different types of macrophages are presented in Table 1. CA macrophages are essential for host defense <sup>84,102</sup> and tumor killing. The pro-inflammatory mediators produced by CA macrophages can cause extensive damage to the host.

AA macrophages seem to play a role in immune suppression and tissue repair, due to production of anti-inflammatory cytokines and extracellular matrix components and in addition failure to produce NO <sup>92</sup>. The most common used distinctive marker for AA macrophages, in mice, is the higher expression and activity of arginase <sup>92</sup>. Due to the activation of arginase, arginine is converted to ornithine, a precursor for polyamines and collagen, which contributes to the production of extracellular matrix <sup>103-106</sup>. The polyamines produced can influence production of cytokines and suppress clonal expansion of lymphocytes, thereby having a regulatory effect on the immune response <sup>107</sup>.

Functional differences can be observed between CA and AA macrophages. As mentioned above, due to the production of ROS, CA macrophages are efficient in the killing of bacteria, while AA macrophages do not produce ROS and are therefore less efficient in killing bacteria <sup>84</sup>. Furthermore, CA macrophages are efficient antigen presenting cells, while AA macrophages are not <sup>92</sup>. CA macrophages are also more efficient in activating T-cell proliferation compared to AA macrophages <sup>92</sup>. AA macrophages are involved in scar formation, since they enhance fibrogenesis, while CA macrophages do not. AA macrophages stimulate proliferation and activation of fibroblasts, by expression and release of potent fibrogenic growth



MARKER	CA-MΦ	AA-MΦ	SPECIES	REF
<b>ENZYMES</b>				
iNOS mRNA expression, activity, NO release	↑	-	Mouse	91, 204
Arginase mRNA expression activity	-	↑	Mouse	91
SPHK1 mRNA	-	-	Mouse	91
12,15-lipoxygenase	↓	↑	Human, mouse	205
<b>MEMBRANE RECEPTOR EXPRESSION</b>				
CD163 mRNA and protein expression	-	↑	Human, mouse	91, 206
Mannose receptor expression	-	↑	Mouse	93
β-glucan receptor (Dectin-1)	-	↑	Mouse	207
MGL1/2 mRNA and protein expression	-	↑	Human, mouse	208
FcγRI	↑	↓	Human	209
LIGHT MRNA	-	-	Mouse	91
E-CADHERIN	-	↑	Human, mouse	99, 210
FACTOR XIIIa	-	↑	Human	211
CCR7	↑	↓	Human	212
<b>ANTIGEN PRESENTATION</b>				
MHC class II protein expression	↑	↓	Human, mouse	91
CD86 protein expression	↑	↓	Human, mouse	91
MS1-HMWP	↓	↑	Human	89
CD40	↑	-	Human	100
<b>CYTOKINE</b>				
IL-12 release	↑	-	Human	91, 213, 214
IL-12 mRNA	↑↑	-	mouse	
IL-10 mRNA and release	-	-	Mouse	91, 214, 215
Ratio IL-10/IL-12	↓	-	Mouse	214, 215
IL-23 release	↑	-	Human	216
IL-6	↑	↓	Mouse	214
TNF	↑	↓	Human	217
IL-1Ra/IL-1 decoy receptor release	-	↑	Mouse	89
<b>CHEMOKINE</b>				
AMAC-1 release	-	↑	Human	218
MIP-1α mRNA	↑	-	Human	89, 218
MDC (CCL22) mRNA expression	-	↑	Human	219
TARC (CCL17)	↓	↑	Human, mouse	220, 221
<b>SECRETORY PROTEINS</b>				
FIZZ1 mRNA	-	↑	Mouse	222
YM1/2 mRNA	-	↑	Mouse	222

Table 1: Markers for the different macrophage subtypes. ↑ an increase in expression/activity, – no change in expression/activity. ↓ : a decrease in expression/activity. ? : unknown.

factors, like transforming growth factor-beta (TGF- $\beta$ ) and platelet derived growth factor (PDGF) <sup>108</sup>. The angiogenic potential of AA macrophages is higher compared to CA macrophages <sup>109</sup>. Due to the production of growth factors and stimulation of angiogenesis, AA macrophages are considered tumor promoting. *In vitro* CA macrophages were shown to be cytotoxic to tumor cells but not to normal cells <sup>110</sup>.

Until now, little research has been done about the presence and function of these different subsets of macrophages in MS.

## MACROPHAGES IN AXONAL DAMAGE

It is generally accepted that macrophages/microglia are involved in the pathogenesis of MS and EAE. Some indirect, though very convincing, evidence has been found supporting a role for macrophages in axonal damage. First of all, a close association has been found between inflammation and neurodegeneration in all lesional and disease stages of MS <sup>111</sup>. Another important finding is the correlation between the location of axonal damage and cellular infiltrates, the locations with the most axonal loss also showed signs of inflammation and macrophage presence <sup>40;48;112</sup>. Furthermore a correlation between the number of infiltrating macrophages and the extent of axonal damage was observed in active and chronic active MS lesions <sup>47;49;112</sup>. Elimination of infiltrating macrophages or resident microglia in the CNS has a suppressive effect on the clinical signs of EAE and reduced CNS inflammation <sup>113;114</sup>. Conversely, activating macrophages by blocking the inhibitory signal of CD200/CD200R between neurons and macrophages, led to a significant enhancement of the clinical signs of EAE <sup>115;116</sup>. In MS lesions a decrease in expression of macrophage inhibitory molecules, CD200 and CD47, was found indicating that release of inhibition of macrophages could play a role in MS <sup>116</sup>. Macrophage and microglial activation is associated with an upregulation of a plethora of inflammatory mediators that could mediate the acute damage seen in the axons even early in the disease. In the following paragraphs these mediators with their possible mechanisms will be shortly reviewed.

### *Oxygen radicals*

During inflammation, as occurs in MS lesions, ROS, such as superoxide and NO, are released in high concentrations. Many studies have shown increased concentrations of markers for oxidative stress, such as oxidized proteins, lipids and DNA, in the CNS of EAE animals and MS patients and sera of MS patients <sup>117-122</sup>. ROS production by activated peripheral blood mononuclear cells is increased in MS patients during a relapse <sup>123;124</sup>. A higher production of NO has also been found in MS lesions, reflected by the expression of iNOS in macrophages/microglia and astrocytes <sup>125;126</sup>. In MS lesions, nitrotyrosine, a marker for peroxynitrite formation, was observed <sup>126;127</sup> and in EAE it was found to correlate with disease severity <sup>128</sup>. In CSF, serum and urine of MS patients increased levels of the metabolites of NO production have been found <sup>129;130</sup>.

ROS play an important role in the pathogenesis of MS and EAE. A strong indication for the role of ROS in EAE was given by the fact that treatment with ROS

scavengers and antioxidants reduced inflammation and axonal damage in acute and chronic EAE <sup>131-134</sup>. ROS can also indirectly induce toxic effects through their ability to inhibit glutamate transporters and thereby inducing excitotoxicity <sup>113;135</sup>. A few possible ways by which ROS induce axonal damage and neuronal death have been proposed. Peroxynitrite can damage both myelin and axons <sup>136</sup>. NO can induce a reversible conduction block in axons exposed to low frequency stimulation <sup>137</sup>. When axons, that are conducting impulses at physiological frequencies, are exposed to levels of NO which are likely to occur at sites of inflammation, they degenerate <sup>138;139</sup>. Furthermore, by inducing oxidative stress in mitochondria, ROS and NO could lead to axonal damage <sup>140-142</sup>. Axons are metabolically very active, which makes them especially sensitive to the effects of extracellular ROS from macrophages. Mitochondrial dysfunction, due to the oxidative stress, leads to energy deficiency and can thereby lead to impairment of axonal transport, accumulation of APP and ultimately neuronal death <sup>122</sup>. In both EAE and MS mitochondrial function has been shown to be impaired <sup>76;143</sup> and to correlate with the presence of macrophages/microglia and production of ROS <sup>144</sup>. Reduced energy levels can also cause increased sodium leakage into the axon and thereby reversal of the operation of the sodium-calcium exchanger, axonal swelling and increased intracellular calcium concentrations, leading to the induction of apoptosis <sup>145</sup>. Treatment with sodium and calcium entry blockers protected axons from this oxidative induced mitochondrial damage <sup>142;146;147</sup>.

### *Cytokines*

Cytokines, low-molecular weight immunoregulatory proteins, can have both pro- and anti-inflammatory properties. They can be produced by a variety of cells in a variety of tissues. During MS a plethora of cytokines is produced <sup>148</sup>. Both pro- and anti-inflammatory cytokines are upregulated, seemingly simultaneously as they are all detected in serum, CSF, and cultured mononuclear cells of MS patients and in lesions in EAE in marmosets. A correlation has been observed between the levels of certain cytokines and disease activity, since tumor necrosis factor-alpha (TNF- $\alpha$ ) and IFN- $\gamma$  were correlated with clinical relapses and TGF- $\beta$ , IL-12p40 and IL-10 are related to remission phase <sup>149-152</sup>. Pro-inflammatory cytokines are thought to be involved in disease pathogenesis, while anti-inflammatory cytokines could be important for disease resolution <sup>148</sup>. Interestingly, specific subpopulations of macrophages/microglia express different cytokines <sup>91</sup>. These subpopulations might correspond to the different activation subtypes of macrophages, i.e. alternatively or classically activated (as described above).

Cytokines have many different functions and in a complex disorder like MS it is not always clear whether they are beneficial or detrimental. For example TNF- $\alpha$  has been shown to be toxic to oligodendrocytes *in vitro* and therefore could induce demyelination. After induction of Wallerian degeneration, a process in which TNF- $\alpha$  has been shown to be involved <sup>153</sup>, the number of preserved axons was higher in TNF- $\alpha$  deficient mice compared to wild-types <sup>154</sup>. Further, moderate overexpression of TNF- $\alpha$  leads to demyelination and axonal damage, similar to that observed in EAE and MS <sup>155</sup>. Moreover, TNF- $\alpha$  mRNA expression has been positively correlated

with the demyelinating activity and negatively correlated with oligodendrocyte integrity in periplaque white matter in MS biopsies<sup>156</sup>. TNF also has protective effects like its elimination of autoreactive T cells via TNF receptor 1<sup>157</sup>. Finally, treatment with anti-TNF- $\alpha$  antibodies was beneficial in EAE. However, treatment in humans with an antibody directed to TNF- $\alpha$  enhanced the disease<sup>113;158;159</sup>. Another indication that cytokines might be differentially involved in MS and EAE came from studies using IFN- $\gamma$ . Treatment with anti-IFN- $\gamma$  led to more severe EAE, while administration of IFN- $\gamma$  ameliorated the disease<sup>160;161</sup>. In humans the opposite occurred, since treatment with IFN- $\gamma$  worsened MS<sup>162</sup>. Similar divergent effects have been described for IL-6. No demyelination and inflammation were found in EAE induced in mice deficient for IL-6, however absence of IL-6 immunoreactivity correlated with oligodendrocyte loss in inactive demyelinated MS lesions. IL-6 might be involved in remyelination through its effects on oligodendrocyte survival, migration and differentiation<sup>163</sup>.

### *Glutamate*

Glutamate is the most common excitatory neurotransmitter in the CNS. However, excessive concentrations of glutamate lead to overstimulation of the glutamate receptors and finally cell death, through influx of calcium. This type of cell death is called excitotoxicity. In EAE excitotoxicity was reported to be of importance since AMPA/kainate receptor antagonists were shown to ameliorate the clinical score, which corresponded pathologically to a reduction in the loss of oligodendrocytes and axonal damage<sup>164-166</sup>. Excitotoxicity is thought to play a role in MS since increased concentrations of glutamate have been observed in CSF of MS patients<sup>167;168</sup> and this increase was found to be associated with the severity and course of the disease<sup>169</sup>. Alterations in glutamate homeostasis, e.g. patterns of expression of glutamate receptors, transporters and glutamate-metabolizing enzymes, have been found in both MS and EAE and were associated with demyelination and axonal damage<sup>170-175</sup>. In lesions of MS patients increased expression of glutaminase has been observed in activated macrophages and microglia near damaged neurons<sup>170</sup>. Macrophages, microglia and astrocytes are sources of excess glutamate in EAE and MS. Furthermore, pro-inflammatory mediators, like IL-1 $\beta$  and TNF- $\alpha$  are suggested to decrease glutamate clearance by astrocytes, increasing extracellular glutamate and enhancing excitotoxicity<sup>176</sup>.

### *Proteases*

Macrophages also release matrix metalloproteinases (MMPs), in order to degrade the extracellular matrix to facilitate migration in the CNS. In MS lesions MMPs are present and they may directly cause axonal transections<sup>177-182</sup>. Macrophage-mediated axonal retraction, as visible in spinal cord injury, has been found to be due to the expression of MMP9<sup>183</sup>. Another protease that plays a role during MS is tissue plasminogen activator (tPA). It is increased in both MS and EAE<sup>184-187</sup>. Microglia and macrophages secrete tPA. Furthermore, tPA is involved in monocyte migration over the blood brain barrier, by the break down of tight junctions between endothelial cells<sup>187</sup>. *In vitro*, tPA is able to induce neuronal apoptosis<sup>188</sup>.

Delayed demyelination and axonal degeneration has been found after induction of EAE in tPA knock out mice <sup>184</sup>.

### MACROPHAGES IN AXONAL REPAIR

Although the studies mentioned above all indicate that macrophages play a detrimental role, the truth is probably not as black and white. Several studies have shown that macrophages could also play beneficial roles during CNS repair. A first indication, suggesting that macrophages actually stimulate regeneration in axons, came from retinal ganglion cell regeneration. An early study found that macrophages appeared to mediate the pro-regenerative effects of lens injury after nerve crush, since macrophage infiltration corresponded with an upregulation in growth-associated protein (GAP)-43 expression levels. Moreover, intraocular zymosan injection, which results in massive macrophage infiltration, led to increased GAP-43 expression and axonal regeneration in absence of lens injury. *In vitro*, zymosan stimulated macrophage conditioned medium was able to enhance axon regeneration, with the axon-promoting effects being mediated by molecules smaller than 30 kD. The molecule responsible for the pro-regenerative effects of activated macrophages was oncomodulin and affected not only retinal ganglion cells but also dorsal root ganglion cells <sup>189</sup>. Moreover, macrophages recruited to the site of nerve crush express the axon guidance molecule EphB3, while injured retinal ganglion cell axons express EphrinB3, a receptor for EphB3. EphB3 stimulated retinal ganglion cell outgrowth toward the source of EphB3 *in vitro*, whereas in heterozygous and homozygous null mutant adult animals a significant decrease in axon outgrowth in the injured nerve could be observed <sup>190</sup>.

In the field of spinal cord injury (SCI), divergent effects of macrophages have been reported as well. After SCI, implantation of macrophages that were pre-exposed *ex vivo* to peripheral nerve fragments induced repair and partial recovery of functionality <sup>191</sup>. During SCI myeloid cells, including macrophages, appeared to be essential for repair since they create a growth-permissive environment in which axonal regeneration can take place <sup>192</sup>. Shechter et al. <sup>193</sup> have found that at the margins of a lesion infiltrating macrophages mediate an immunoregulatory role by secreting the anti-inflammatory cytokine IL-10, which contributes to recovery after SCI. The release of IL-10 indicates that these beneficial macrophages could have an AA macrophage phenotype, since AA macrophages secrete high levels of IL-10 <sup>92</sup>. Finally, Kigerl et al. <sup>194</sup> have identified both CA and AA macrophages during SCI in the mouse. They found that the CA macrophage response persisted, while AA macrophage response was transient, which could lead to the stunted functional recovery seen in these animals. *In vitro* these authors showed that CA macrophages are neurotoxic, while AA macrophages were not and could actually induce neuronal outgrowth even on a growth inhibiting substrate.

Recent evidence also points to a beneficial role of macrophages during repair in MS. Depletion of macrophages has been shown to reduce remyelination in demyelinating models <sup>195,196</sup>, due to reduced oligodendrocyte progenitor recruitment and differentiation and altered growth factor expression <sup>196</sup>. Conversely, enrichment of whole brain aggregate cultures with macrophages promotes the capacity for

initial myelination and remyelination following demyelination<sup>197</sup>. Moreover, through the removal of myelin debris and stimulation of oligodendrocyte progenitor migration, proliferation and differentiation, macrophages can promote remyelination. Activated macrophages/microglia are also a source of a variety of growth factors, neurotrophins and their receptors during EAE and in MS lesions<sup>198-200</sup>.

Macrophages could contribute to the resolution of the inflammation in MS thereby inhibiting further injury to the axons. It was found that myelin-laden foamy macrophages in active lesions expressed anti-inflammatory molecules, while pro-inflammatory molecules were not expressed. *In vitro*, myelin ingestion induced foamy macrophage morphology and expression of anti-inflammatory molecules and inhibited the response to pro-inflammatory stimuli. This indicated a strong immunosuppressive function for foamy macrophages<sup>201</sup>. These foamy macrophages display functions and activities that might put them in the category of AA macrophages, since they produced anti-inflammatory cytokines and showed suppressed LPS activation *in vitro*. Another indication that foamy macrophages might be AA macrophages was that they express CD163, a marker for AA macrophages, although the expression of mannose receptor, another marker for AA macrophages, is low in these cells<sup>202</sup>. Furthermore, perivascular macrophages, which are located at the blood brain barrier, have an AA macrophage phenotype, since they do express both CD163 and mannose receptor. This could be important since their location at the blood brain barrier means they occupy a strategic position to control innate and adaptive immune responses in the brain. The AA macrophage phenotype might be responsible for a lower inflammation rate at the blood brain barrier.

Another indication that macrophages are involved in axonal repair was found in the fact that activated macrophages are present in the areas of increased GAP-43 expression. Levels of GAP-43, a marker for axonal growth and synaptogenesis, were decreased inside lesions, while being upregulated around lesions. Although no correlation was found between the intensity of GAP-43 staining and macrophage presence, macrophages were consistently observed in areas of increased GAP-43 expression. Macrophages could be the source of neurotrophic factors that increase GAP-43 expression<sup>203</sup>. However, the authors did not investigate the phenotype of the macrophages present in the areas of GAP-43 expression in comparison to the macrophages present inside the lesions.

## AIM OF THE THESIS

As discussed above, activated macrophages can have both beneficial and detrimental effects in the development of MS lesions. These divergent effects of macrophages could be due to the fact that different subtypes or activation phenotypes of macrophages exist (hypothesis depicted in Figure 1). In the CNS and specifically in MS relatively little research has been done focusing on the divergent effects of the different subtypes of macrophages. In this thesis the aim was to determine the effects of differently activated macrophages on axonal damage and repair.

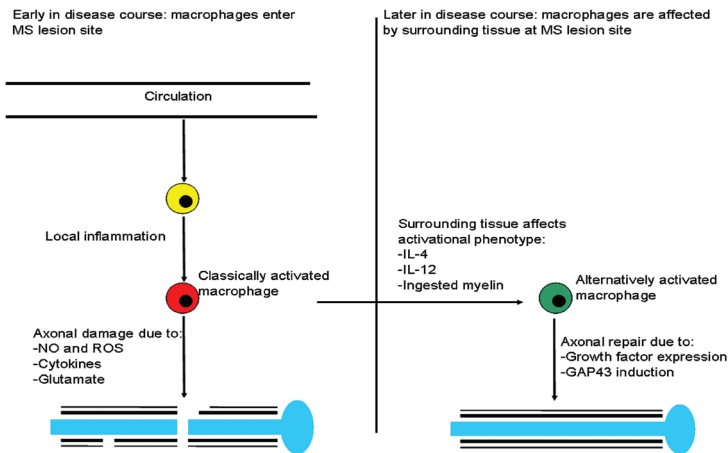


Figure 1: Hypothesis of macrophage activation in MS lesions. As the macrophages enter the lesion site, they become classically activated due to the local inflammation. These classically activated macrophages induce axonal damage by secreting neurotoxic substances such as NO, pro-inflammatory cytokines and glutamate. The macrophages at the lesion are present in the CNS tissue for longer periods of time. Slowly the surrounding tissue starts to affect the activational phenotype of the macrophages. Due to IL-12 and IL-4 secreted by astrocytes and ingestion of myelin, the macrophages take on an alternatively activated phenotype. These macrophages are involved in axonal repair due to the expression of growth factors, induction of GAP-43 expression in neurons and secretion of anti-inflammatory cytokines.

## Outline

The main focus of this thesis was to investigate the role of phenotypically different macrophages on various phases of axonal damage during MS. Whole brain spheroid cultures provide a valuable model in which to study the effects of macrophages on neuronal damage during de- and remyelination. In the spheroid cultures all CNS cells are present in a three dimensional conformation and multilayered myelin is formed. Axons are myelinated in spheroids, in contrast to MS lesions where the myelin sheath is damaged. Therefore, in order to mimic the demyelinated state in the spheroid model we aimed to develop a model to induce demyelination in the spheroids (**chapter 2**).

Little research has been performed on differently activated macrophages in the CNS. First we determined the migratory characteristics of differently activated macrophages in the context of the CNS. We studied the migration of AA and CA macrophages towards conditioned medium from different CNS cell types and the motility and adhesion of AA and CA macrophages (**chapter 3**).

Next, we addressed the question how the AA phenotype is induced in MS lesions. Boven et al. 201 had suggested that in MS lesions foamy macrophages have a AA phenotype, since they expressed little pro-inflammatory cytokines and iNOS. *In vitro*, myelin ingestion was found to inhibit the LPS response. Liver X receptor (LXR) is involved in both lipid metabolism and immune modulation. We hypothesized that activation of liver X receptor (LXR) by myelin ingestion could lead to the AA

phenotype and reduction in LPS response due to the blocking effect of LXR on nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B). In **chapter 4** we investigated whether knock-down of LXR could inhibit the repression of the LPS response seen after myelin ingestion. However, during this study we observed differences between myelin preparations in cytokine induction. One preparation induced the expression of IL-10 and TNF- $\alpha$  and LPS insensitivity, while another did not. We questioned whether LPS contamination in one of the preparations was responsible for induction of both the high anti-inflammatory cytokine levels and the LPS insensitivity (**chapter 5**).

**Chapters 6 and 7** focus on the effects of differently activated murine macrophages on neurons. In MS lesions the most prominent function of macrophages is the phagocytosis of myelin. However, due to axonal damage, neuronal debris will also be present in lesions. Therefore we aimed to determine whether the phagocytosis and degradation of neurons and neuronal fragments differs between the different subtypes of macrophages (**chapter 6**). We next investigated whether the differently activated macrophages could have divergent effects on neuronal cultures. We exposed neuronal cultures directly to differently activated macrophages or their conditioned medium. We hypothesized that CA macrophages would be damaging to neurons, due to the release of cytotoxic agents and the phagocytosis of neurons, and that AA macrophages could be neuroprotective (**chapter 7**).

In **Chapter 8** we focus on the presence of CA and AA macrophages in MS lesions. We determined whether these divergent phenotypes actually occurred in MS lesions (chapter 8). Indications that differently activated macrophages were present in MS lesions had been found, but until now nobody had ever performed a systematic study of markers for both CA and AA macrophages in MS lesions. We selected a panel of markers based on literature that should be differently expressed on human AA versus CA macrophages and determined their expression in lesions.

## REFERENCE LIST

1. Pugliatti, M., S. Sotgiu, and G. Rosati. 2002. The worldwide prevalence of multiple sclerosis. *Clin. Neurol. Neurosurg.* 104:182-191.
2. Compston, A. and A. Coles. 2008. Multiple sclerosis. *Lancet* 372:1502-1517.
3. McDonald, W.I., A. Compston, G. Edan, D. Goodkin, H.P. Hartung, F.D. Lublin, H.F. McFarland, D.W. Paty, C.H. Polman, S.C. Reingold, M. Sandberg-Wollheim, W. Sibley, A. Thompson, van den Noort S., B.Y. Weinstenker, and J.S. Wolinsky. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50:121-127.
4. Barkhof, F., M. Filippi, D.H. Miller, P. Scheltens, A. Campi, C.H. Polman, G. Comi, H.J. Ader, N. Losseff, and J. Valk. 1997. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* 120 (Pt 11):2059-2069.
5. Nielsen, J.M., T. Korteweg, F. Barkhof, B.M. Uitdehaag, and C.H. Polman. 2005. Overdiagnosis of multiple sclerosis and magnetic resonance imaging criteria. *Ann. Neurol.* 58:781-783.
6. Polman, C.H., S.C. Reingold, G. Edan, M. Filippi, H.P. Hartung, L. Kappos, F.D. Lublin, L.M. Metz, H.F. McFarland, P.W. O'Connor, M. Sandberg-Wollheim, A.J. Thompson,



- B.G.Weinshenker, and J.S.Wolinsky. 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the “McDonald Criteria”. *Ann. Neurol.* 58:840-846.
7. Bruck,W., A.Bitsch, H.Kolenda, Y.Bruck, M.Stiefel, and H.Lassmann. 1997. Inflammatory central nervous system demyelination: correlation of magnetic resonance imaging findings with lesion pathology. *Ann. Neurol.* 42:783-793.
  8. Miller,D.H., R.I.Grossman, S.C.Reingold, and H.F.McFarland. 1998. The role of magnetic resonance techniques in understanding and managing multiple sclerosis. *Brain* 121 ( Pt 1):3-24.
  9. Nesbit,G.M., G.S.Forbes, B.W.Scheithauer, H.Okazaki, and M.Rodriguez. 1991. Multiple sclerosis: histopathologic and MR and/or CT correlation in 37 cases at biopsy and three cases at autopsy. *Radiology* 180:467-474.
  10. Katz,D., J.K.Taubenberger, B.Cannella, D.E.McFarlin, C.S.Raine, and H.F.McFarland. 1993. Correlation between magnetic resonance imaging findings and lesion development in chronic, active multiple sclerosis. *Ann. Neurol.* 34:661-669.
  11. Correale,J. and de los Milagros Bassani Molinas. 2002. Oligoclonal bands and antibody responses in multiple sclerosis. *J. Neurol.* 249:375-389.
  12. Bourahoui,A., De Seze J., R.Gutierrez, B.Onraed, B.Hennache, D.Feriby, T.Stojkovic, and P.Vermersch. 2004. CSF isoelectrofocusing in a large cohort of MS and other neurological diseases. *Eur. J. Neurol.* 11:525-529.
  13. Paolino,E., E.Fainardi, P.Ruppi, M.R.Tola, V.Govoni, I.Casetta, V.C.Monetti, E.Granieri, and M.Carreras. 1996. A prospective study on the predictive value of CSF oligoclonal bands and MRI in acute isolated neurological syndromes for subsequent progression to multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* 60:572-575.
  14. Lublin,F.D. and S.C.Reingold. 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* 46:907-911.
  15. Charcot,M. 1868. Histologie de le sclerose en plaques. *Gaz Hop* 141:554-558.
  16. van der Valk and C.J.De Groot. 2000. Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. *Neuropathol. Appl. Neurobiol.* 26:2-10.
  17. de Groot,C.J., E.Bergers, W.Kamphorst, R.Ravid, C.H.Polman, F.Barkhof, and Van der Valk P. 2001. Post-mortem MRI-guided sampling of multiple sclerosis brain lesions: increased yield of active demyelinating and (p)reactive lesions. *Brain* 124:1635-1645.
  18. Bruck,W., P.Porada, S.Poser, P.Rieckmann, F.Hanefeld, H.A.Kretzschmar, and H.Lassmann. 1995. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann. Neurol.* 38:788-796.
  19. van der Goes A., W.Boorsma, K.Hoekstra, L.Montagne, C.J.De Groot, and C.D.Dijkstra. 2005. Determination of the sequential degradation of myelin proteins by macrophages. *J. Neuroimmunol.* 161:12-20.
  20. Wolswijk,G. 2000. Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis. *Brain* 123 ( Pt 1):105-115.
  21. Rodriguez,M. and C.F.Lucchinetti. 1999. Is apoptotic death of the oligodendrocyte a critical event in the pathogenesis of multiple sclerosis? *Neurology* 53:1615-1616.
  22. Barnett,M.H. and J.W.Prineas. 2004. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann. Neurol.* 55:458-468.
  23. Henderson,A.P., M.H.Barnett, J.D.Parratt, and J.W.Prineas. 2009. Multiple sclerosis: distribution of inflammatory cells in newly forming lesions. *Ann. Neurol.* 66:739-753.

24. Franklin,R.J. 2002. Why does remyelination fail in multiple sclerosis? *Nat. Rev. Neurosci.* 3:705-714.
25. Bruck,W., T.Kuhlmann, and C.Stadelmann. 2003. Remyelination in multiple sclerosis. *J. Neurol. Sci.* 206:181-185.
26. Prineas,J.W., R.O.Barnard, E.E.Kwon, L.R.Sharer, and E.S.Cho. 1993. Multiple sclerosis: remyelination of nascent lesions. *Ann. Neurol.* 33:137-151.
27. Prineas,J.W. and F.Connell. 1979. Remyelination in multiple sclerosis. *Ann. Neurol.* 5:22-31.
28. Raine,C.S. and E.Wu. 1993. Multiple sclerosis: remyelination in acute lesions. *J. Neuropathol. Exp. Neurol.* 52:199-204.
29. Lassmann,H., W.Bruck, C.Lucchinetti, and M.Rodriguez. 1997. Remyelination in multiple sclerosis. *Mult. Scler.* 3:133-136.
30. Wolswijk,G. 2002. Oligodendrocyte precursor cells in the demyelinated multiple sclerosis spinal cord. *Brain* 125:338-349.
31. John,G.R., S.L.Shankar, B.Shafit-Zagardo, A.Massimi, S.C.Lee, C.S.Raine, and C.F.Brosnan. 2002. Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat. Med.* 8:1115-1121.
32. Charles,P., R.Reynolds, D.Seilhean, G.Rougon, M.S.Aigrot, A.Niezgoda, B.Zalc, and C.Lubetzki. 2002. Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? *Brain* 125:1972-1979.
33. Bjartmar,C. and B.D.Trapp. 2001. Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr. Opin. Neurol.* 14:271-278.
34. Lassmann H. 1998. Pathology of Multiple Sclerosis. *In* McAlpine's Multiple Sclerosis. Livingstone C., editor. 323-358.
35. Davie,C.A., G.J.Barker, S.Webb, P.S.Tofts, A.J.Thompson, A.E.Harding, W.I.McDonald, and D.H.Miller. 1995. Persistent functional deficit in multiple sclerosis and autosomal dominant cerebellar ataxia is associated with axon loss. *Brain* 118 ( Pt 6):1583-1592.
36. De Stefano N., S.Narayanan, G.S.Francis, R.Arnaoutelis, M.C.Tartaglia, J.P.Antel, P.M.Matthews, and D.L.Arnold. 2001. Evidence of axonal damage in the early stages of multiple sclerosis and its relevance to disability. *Arch. Neurol.* 58:65-70.
37. Wujek,J.R., C.Bjartmar, E.Richer, R.M.Ransohoff, M.Yu, V.K.Tuohy, and B.D.Trapp. 2002. Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J. Neuropathol. Exp. Neurol.* 61:23-32.
38. Bjartmar,C., G.Kidd, S.Mork, R.Rudick, and B.D.Trapp. 2000. Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. *Ann. Neurol.* 48:893-901.
39. Trapp,B.D., J.Peterson, R.M.Ransohoff, R.Rudick, S.Mork, and L.Bo. 1998. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 338:278-285.
40. Kuhlmann,T., G.Lingfeld, A.Bitsch, J.Schuchardt, and W.Bruck. 2002. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* 125:2202-2212.
41. Filippi,M., M.Bozzali, M.Rovaris, O.Gonen, C.Kesavadas, A.Ghezzi, V.Martinelli, R.I.Grossman, G.Scotti, G.Comi, and A.Falini. 2003. Evidence for widespread axonal damage at the earliest clinical stage of multiple sclerosis. *Brain* 126:433-437.
42. Lovas,G., N.Szilagyi, K.Majtenyi, M.Palkovits, and S.Komoly. 2000. Axonal changes in chronic demyelinated cervical spinal cord plaques. *Brain* 123 ( Pt 2):308-317.
43. Gentleman,S.M., M.J.Nash, C.J.Sweeting, D.I.Graham, and G.W.Roberts. 1993.

- Beta-amyloid precursor protein (beta APP) as a marker for axonal injury after head injury. *Neurosci. Lett.* 160:139-144.
44. Sherriff, F.E., L.R. Bridges, and S. Sivaloganathan. 1994. Early detection of axonal injury after human head trauma using immunocytochemistry for beta-amyloid precursor protein. *Acta Neuropathol.* 87:55-62.
  45. Young, O.S., C.D. Chen, and C.R. Abraham. 2010. Cell-type Dependent Modulation of Notch Signaling by the Amyloid Precursor Protein. *J. Neurochem.*
  46. Koo, E.H., S.S. Sisodia, D.R. Archer, L.J. Martin, A. Weidemann, K. Beyreuther, P. Fischer, C.L. Masters, and D.L. Price. 1990. Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc. Natl. Acad. Sci. U. S. A.* 87:1561-1565.
  47. Ferguson, B., M.K. Matyszak, M.M. Esiri, and V.H. Perry. 1997. Axonal damage in acute multiple sclerosis lesions. *Brain* 120 ( Pt 3):393-399.
  48. Bitsch, A., J. Schuchardt, S. Bunkowski, T. Kuhlmann, and W. Bruck. 2000. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* 123 ( Pt 6):1174-1183.
  49. Trapp, B.D., J. Peterson, R.M. Ransohoff, R. Rudick, S. Mork, and L. Bo. 1998. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 338:278-285.
  50. Casanova, B., M.C. Martinez-Bisbal, C. Valero, B. Celda, L. Marti-Bonmati, A. Pascual, L. Landente, and F. Coret. 2003. Evidence of Wallerian degeneration in normal appearing white matter in the early stages of relapsing-remitting multiple sclerosis: a HMRS study. *J. Neurol.* 250:22-28.
  51. Zhu, B., L. Luo, G.R. Moore, D.W. Paty, and M.S. Cynader. 2003. Dendritic and synaptic pathology in experimental autoimmune encephalomyelitis. *Am. J. Pathol.* 162:1639-1650.
  52. Craner, M.J., A.C. Lo, J.A. Black, and S.G. Waxman. 2003. Abnormal sodium channel distribution in optic nerve axons in a model of inflammatory demyelination. *Brain* 126:1552-1561.
  53. Craner, M.J., B.C. Hains, A.C. Lo, J.A. Black, and S.G. Waxman. 2004. 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.
  54. Nijeholt, G.J., M.A. van Walderveen, J.A. Castelijns, J.H. van Waesberghe, C. Polman, P. Scheltens, P.F. Rosier, P.J. Jongen, and F. Barkhof. 1998. Brain and spinal cord abnormalities in multiple sclerosis. Correlation between MRI parameters, clinical subtypes and symptoms. *Brain* 121 ( Pt 4):687-697.
  55. Rudick, R.A., E. Fisher, J.C. Lee, J. Simon, and L. Jacobs. 1999. Use of the brain parenchymal fraction to measure whole brain atrophy in relapsing-remitting MS. Multiple Sclerosis Collaborative Research Group. *Neurology* 53:1698-1704.
  56. Miller, D.H., F. Barkhof, J.A. Frank, G.J. Parker, and A.J. Thompson. 2002. Measurement of atrophy in multiple sclerosis: pathological basis, methodological aspects and clinical relevance. *Brain* 125:1676-1695.
  57. Bermel, R.A., J. Sharma, C.W. Tjoa, S.R. Puli, and R. Bakshi. 2003. A semiautomated measure of whole-brain atrophy in multiple sclerosis. *J. Neurol. Sci.* 208:57-65.
  58. Simon, J.H., L.D. Jacobs, M.K. Campion, R.A. Rudick, D.L. Cookfair, R.M. Herndon, J.R. Richert, A.M. Salazar, J.S. Fischer, D.E. Goodkin, N. Simonian, M. Lajaunie, D.E. Miller, K. Wende, A. Martens-Davidson, R.P. Kinkel, F.E. Munschauer, III, and C.M. Brownschidle. 1999. A longitudinal study of brain atrophy in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Neurology* 53:139-148.
  59. Kenzie-Graham, A., M.R. Tinsley, K.P. Shah, C. Aguilar, L.V. Strickland, J. Boline, M. Martin, L. Morales, D.W. Shattuck, R.E. Jacobs, R.R. Voskuhl, and A.W. Toga.

2006. Cerebellar cortical atrophy in experimental autoimmune encephalomyelitis. *Neuroimage*. 32:1016-1023.
60. Matthews,P.M., E.Pioro, S.Narayanan, De Stefano N., L.Fu, G.Francis, J.Antel, C.Wolfson, and D.L.Arnold. 1996. Assessment of lesion pathology in multiple sclerosis using quantitative MRI morphometry and magnetic resonance spectroscopy. *Brain* 119 ( Pt 3):715-722.
  61. Simmons,M.L., C.G.Frondoza, and J.T.Coyle. 1991. Immunocytochemical localization of N-acetyl-aspartate with monoclonal antibodies. *Neuroscience* 45:37-45.
  62. De Stefano N., P.M.Matthews, L.Fu, S.Narayanan, J.Stanley, G.S.Francis, J.P.Antel, and D.L.Arnold. 1998. Axonal damage correlates with disability in patients with relapsing-remitting multiple sclerosis. Results of a longitudinal magnetic resonance spectroscopy study. *Brain* 121 ( Pt 8):1469-1477.
  63. Fu,L., P.M.Matthews, De Stefano N., K.J.Worsley, S.Narayanan, G.S.Francis, J.P.Antel, C.Wolfson, and D.L.Arnold. 1998. Imaging axonal damage of normal-appearing white matter in multiple sclerosis. *Brain* 121 ( Pt 1):103-113.
  64. Bitsch,A., H.Bruhn, V.Vougioukas, A.Stringaris, H.Lassmann, J.Frahm, and W.Bruck. 1999. Inflammatory CNS demyelination: histopathologic correlation with in vivo quantitative proton MR spectroscopy. *AJNR Am. J. Neuroradiol.* 20:1619-1627.
  65. Lee,M.A., A.M.Blamire, S.Pendlebury, K.H.Ho, K.R.Mills, P.Styles, J.Palace, and P.M.Matthews. 2000. Axonal injury or loss in the internal capsule and motor impairment in multiple sclerosis. *Arch. Neurol.* 57:65-70.
  66. De Stefano N., P.M.Matthews, J.P.Antel, M.Preul, G.Francis, and D.L.Arnold. 1995. Chemical pathology of acute demyelinating lesions and its correlation with disability. *Ann. Neurol.* 38:901-909.
  67. Jasperse,M.M., C.Jakobs, M.J.Eikelenboom, J.M.Nielsen, F.Barkhof, and C.D.Dijkstra. 2007. N-Acetylaspartic acid in cerebrospinal fluid of multiple sclerosis patients determined by gas chromatography-mass spectrometry. *J.Neurol.* In press.
  68. Teunissen,C.E., E.Iacobaeus, M.Khademi, L.Brundin, N.Norgren, M.J.Koel-Simmelink, M.Schepens, F.Bouwman, H.A.Twaalfhoven, H.J.Blom, C.Jakobs, and C.D.Dijkstra. 2009. Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis. *Neurology* 72:1322-1329.
  69. Teunissen,C.E., C.Dijkstra, and C.Polman. 2005. Biological markers in CSF and blood for axonal degeneration in multiple sclerosis. *Lancet Neurol.* 4:32-41.
  70. Yin,X., T.O.Crawford, J.W.Griffin, P.Tu, V.M.Lee, C.Li, J.Roder, and B.D.Trapp. 1998. Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. *J. Neurosci.* 18:1953-1962.
  71. Li,C., M.B.Tropak, R.Gerlai, S.Clapoff, W.bramow-Newerly, B.Trapp, A.Peterson, and J.Roder. 1994. Myelination in the absence of myelin-associated glycoprotein. *Nature* 369:747-750.
  72. Klugmann,M., M.H.Schwab, A.Puhlhofer, A.Schneider, F.Zimmermann, I.R.Griffiths, and K.A.Nave. 1997. Assembly of CNS myelin in the absence of proteolipid protein. *Neuron* 18:59-70.
  73. Griffiths,I., M.Klugmann, T.Anderson, D.Yool, C.Thomson, M.H.Schwab, A.Schneider, F.Zimmermann, M.McCulloch, N.Nadon, and K.A.Nave. 1998. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science* 280:1610-1613.
  74. Lappe-Siefke,C., S.Goebbels, M.Gravel, E.Nicksch, J.Lee, P.E.Braun, I.R.Griffiths, and K.A.Nave. 2003. Disruption of *Cnp1* uncouples oligodendroglial functions in axonal support and myelination. *Nat. Genet.* 33:366-374.

75. Craner, M.J., J. Newcombe, J.A. Black, C. Hartle, M.L. Cuzner, and S.G. Waxman. 2004. Molecular changes in neurons in multiple sclerosis: altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. *Proc. Natl. Acad. Sci. U. S. A* 101:8168-8173.
76. Witte, M.E., L. Bo, R.J. Rodenburg, J.A. Belien, R. Musters, T. Hazes, L.T. Wintjes, J.A. Smeitink, J.J. Geurts, H.E. de Vries, Van der Valk P., and van Horsen J. 2009. Enhanced number and activity of mitochondria in multiple sclerosis lesions. *J. Pathol.* 219:193-204.
77. Qi, X., A.S. Lewin, L. Sun, W.W. Hauswirth, and J. Guy. 2006. Mitochondrial protein nitration primes neurodegeneration in experimental autoimmune encephalomyelitis. *J. Biol. Chem.* 281:31950-31962.
78. Medzhitov, R. and C.A. Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296:298-300.
79. Janeway, C.A., Jr. and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197-216.
80. Takeda, K. and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17:1-14.
81. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.
82. DeLeo, F.R., L.A. Allen, M. Apicella, and W.M. Nauseef. 1999. NADPH oxidase activation and assembly during phagocytosis. *J. Immunol.* 163:6732-6740.
83. Babior, B.M. 1999. NADPH oxidase: an update. *Blood* 93:1464-1476.
84. Gordon, S. and P.R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5:953-964.
85. Gordon, S. 1986. Biology of the macrophage. *J. Cell Sci. Suppl* 4:267-286.
86. Gordon, S. 1998. The role of the macrophage in immune regulation. *Res Immunol.* 149:685-688.
87. Kono, H. and K.L. Rock. 2008. How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* 8:279-289.
88. Zhang, X. and D.M. Mosser. 2008. Macrophage activation by endogenous danger signals. *J. Pathol.* 214:161-178.
89. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3:23-35.
90. Mosser, D.M. 2003. The many faces of macrophage activation. *J. Leukoc. Biol.* 73:209-212.
91. Mosser, D.M. and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8:958-969.
92. Edwards, J.P., X. Zhang, K.A. Frauwirth, and D.M. Mosser. 2006. Biochemical and functional characterization of three activated macrophage populations. *J. Leukoc. Biol.* 80:1298-1307.
93. Martinez, F.O., A. Sica, A. Mantovani, and M. Locati. 2008. Macrophage activation and polarization. *Front Biosci.* 13:453-461.
94. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potentially enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176:287-292.
95. Modollell, M., I.M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol.* 25:1101-1104.
96. Nathan, C. and M.U. Shiloh. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Natl. Acad. Sci. U. S. A* 97:8841-8848.

97. O'Shea, J.J. and P.J. Murray. 2008. Cytokine signaling modules in inflammatory responses. *Immunity*. 28:477-487.
98. MacMicking, J., Q.W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15:323-350.
99. Hibbs, J.B., Jr. 2002. Infection and nitric oxide. *J. Infect. Dis.* 185 Suppl 1:S9-17.
100. Van den Bossche J., P. Bogaert, H.J. van, C.J. Guerin, G. Berx, K. Movahedi, Van den Bergh R., A. Pereira-Fernandes, J.M. Geuns, H. Pircher, P. Dorny, J. Grooten, De Baetselier P., and J.A. Van Ginderachter. 2009. Alternatively activated macrophages engage in homotypic and heterotypic interactions through IL-4 and polyamine-induced E-cadherin/catenin complexes. *Blood* 114:4664-4674.
101. Zeyda, M., D. Farmer, J. Todoric, O. Aszmann, M. Speiser, G. Gyori, G.J. Zlabinger, and T.M. Stulnig. 2007. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int. J. Obes. (Lond)* 31:1420-1428.
102. Nathan, C. 2008. Metchnikoff's Legacy in 2008. *Nat. Immunol.* 9:695-698.
103. Kreider, T., R.M. Anthony, J.F. Urban, Jr., and W.C. Gause. 2007. Alternatively activated macrophages in helminth infections. *Curr. Opin. Immunol.* 19:448-453.
104. Albina, J.E., C.D. Mills, W.L. Henry, Jr., and M.D. Caldwell. 1990. Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.* 144:3877-3880.
105. Gratchev, A., P. Guillot, N. Hakiy, O. Politz, C.E. Orfanos, K. Schledzewski, and S. Goerd. 2001. Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein beta1G-H3. *Scand. J. Immunol.* 53:386-392.
106. Hesse, M., M. Modolell, A.C. La Flamme, M. Schito, J.M. Fuentes, A.W. Cheever, E.J. Pearce, and T.A. Wynn. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J. Immunol.* 167:6533-6544.
107. Cordeiro-da-Silva, A., J. Tavares, N. Araujo, F. Cerqueira, A. Tomas, L.P. Kong Thoo, and A. Ouassii. 2004. Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells. *Int. Immunopharmacol.* 4:547-556.
108. Song, E., N. Ouyang, M. Horbelt, B. Antus, M. Wang, and M.S. Exton. 2000. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol.* 204:19-28.
109. Kodolja, V., C. Muller, S. Tenorio, C. Schebesch, C.E. Orfanos, and S. Goerd. 1997. Differences in angiogenic potential of classically vs alternatively activated macrophages. *Immunobiology* 197:478-493.
110. Romieu-Mourez, R., M. Solis, A. Nardin, D. Goubau, V. Baron-Bodo, R. Lin, B. Massie, M. Salcedo, and J. Hiscott. 2006. Distinct roles for IFN regulatory factor (IRF)-3 and IRF-7 in the activation of antitumor properties of human macrophages. *Cancer Res* 66:10576-10585.
111. Frischer, J.M., S. Bramow, A. Dal-Bianco, C.F. Lucchinetti, H. Rauschka, M. Schmidbauer, H. Laursen, P.S. Sorensen, and H. Lassmann. 2009. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 132:1175-1189.
112. Kornek, B., M.K. Storch, R. Weissert, E. Wallstroem, A. Stefferl, T. Olsson, C. Linington, M. Schmidbauer, and H. Lassmann. 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.
113. Hendriks, J.J., C.E. Teunissen, H.E. de Vries, and C.D. Dijkstra. 2005. Macrophages

- and neurodegeneration. *Brain Res. Brain Res. Rev.* 48:185-195.
114. Heppner, F.L., M. Greter, D. Marino, J. Falsig, G. Raivich, N. Hovelmeyer, A. Waisman, T. Rulicke, M. Prinz, J. Priller, B. Becher, and A. Aguzzi. 2005. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med.* 11:146-152.
  115. Meuth, S.G., O.J. Simon, A. Grimm, N. Melzer, A.M. Herrmann, P. Spitzer, P. Landgraf, and H. Wiendl. 2008. CNS inflammation and neuronal degeneration is aggravated by impaired CD200-CD200R-mediated macrophage silencing. *J. Neuroimmunol.* 194:62-69.
  116. Koning, N., L. Bo, R.M. Hoek, and I. Huitinga. 2007. Downregulation of macrophage inhibitory molecules in multiple sclerosis lesions. *Ann. Neurol.* 62:504-514.
  117. Naidoo, R. and M.L. Knapp. 1992. Studies of lipid peroxidation products in cerebrospinal fluid and serum in multiple sclerosis and other conditions. *Clin. Chem.* 38:2449-2454.
  118. Besler, H.T. and S. Comoglu. 2003. Lipoprotein oxidation, plasma total antioxidant capacity and homocysteine level in patients with multiple sclerosis. *Nutr. Neurosci.* 6:189-196.
  119. MacMicking, J.D., D.O. Willenborg, M.J. Weidemann, K.A. Rockett, and W.B. Cowden. 1992. Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J. Exp. Med.* 176:303-307.
  120. Toshniwal, P.K. and E.J. Zarling. 1992. Evidence for increased lipid peroxidation in multiple sclerosis. *Neurochem. Res* 17:205-207.
  121. Vladimirova, O., J.O'Connor, A. Cahill, H. Alder, C. Butunoi, and B. Kalman. 1998. Oxidative damage to DNA in plaques of MS brains. *Mult. Scler.* 4:413-418.
  122. Lu, F., M. Selak, J.O'Connor, S. Croul, C. Lorenzana, C. Butunoi, and B. Kalman. 2000. Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. *J. Neurol. Sci.* 177:95-103.
  123. Vladimirova, O., F.M. Lu, L. Shawver, and B. Kalman. 1999. The activation of protein kinase C induces higher production of reactive oxygen species by mononuclear cells in patients with multiple sclerosis than in controls. *Inflamm. Res* 48:412-416.
  124. Ferretti, G., T. Bacchetti, F. Di Ludovico, B. Viti, V.A. Angeleri, M. Danni, and L. Provinciali. 2006. Intracellular oxidative activity and respiratory burst of leukocytes isolated from multiple sclerosis patients. *Neurochem. Int.* 48:87-92.
  125. De Groot, C.J., S.R. Ruuls, J.W. Theeuwes, C.D. Dijkstra, and Van der Valk P. 1997. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* 56:10-20.
  126. Bagasra, O., F.H. Michaels, Y.M. Zheng, L.E. Bobroski, S.V. Spitsin, Z.F. Fu, R. Tawadros, and H. Koprowski. 1995. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A* 92:12041-12045.
  127. Liu, J.S., M.L. Zhao, C.F. Brosnan, and S.C. Lee. 2001. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am. J. Pathol.* 158:2057-2066.
  128. Van der Veen R.C., D.R. Hinton, F. Incardonna, and F.M. Hofman. 1997. Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. *J. Neuroimmunol.* 77:1-7.
  129. Giovannoni, G., N.C. Silver, J.O'Riordan, R.F. Miller, S.J. Heales, J.M. Land, M. Elliot, M. Feldmann, D.H. Miller, and E.J. Thompson. 1999. Increased urinary nitric oxide

- metabolites in patients with multiple sclerosis correlates with early and relapsing disease. *Mult. Scler.* 5:335-341.
130. Giovannoni,G. 1998. Cerebrospinal fluid and serum nitric oxide metabolites in patients with multiple sclerosis. *Mult. Scler.* 4:27-30.
  131. Hendriks,J.J., J.Alblas, S.M.van der Pol, E.A.van Tol, C.D.Dijkstra, and H.E.de Vries. 2004. Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.* 200:1667-1672.
  132. Liu,Y., B.Zhu, X.Wang, L.Luo, P.Li, D.W.Paty, and M.S.Cynader. 2003. Bilirubin as a potent antioxidant suppresses experimental autoimmune encephalomyelitis: implications for the role of oxidative stress in the development of multiple sclerosis. *J. Neuroimmunol.* 139:27-35.
  133. Marracci,G.H., R.E.Jones, G.P.McKeon, and D.N.Bourdette. 2002. Alpha lipoic acid inhibits T cell migration into the spinal cord and suppresses and treats experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 131:104-114.
  134. Schreibelt,G., R.J.Musters, A.Reijkerk, L.R.de Groot, S.M.van der Pol, E.M.Hendriks, E.D.Dopp, C.D.Dijkstra, B.Drukarch, and H.E.de Vries. 2006. Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J. Immunol.* 177:2630-2637.
  135. Gilgun-Sherki,Y., E.Melamed, and D.Offen. 2004. The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J. Neurol.* 251:261-268.
  136. Touil,T., M.S.oire-Grassin, C.Vital, K.G.Petry, and B.Brochet. 2001. In vivo damage of CNS myelin and axons induced by peroxynitrite. *Neuroreport* 12:3637-3644.
  137. Redford,E.J., R.Kapoor, and K.J.Smith. 1997. Nitric oxide donors reversibly block axonal conduction: demyelinated axons are especially susceptible. *Brain* 120 ( Pt 12):2149-2157.
  138. Smith,K.J. and H.Lassmann. 2002. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* 1:232-241.
  139. Smith,K.J., R.Kapoor, S.M.Hall, and M.Davies. 2001. Electrically active axons degenerate when exposed to nitric oxide. *Ann. Neurol.* 49:470-476.
  140. Brown,G.C., J.P.Bolanos, S.J.Heales, and J.B.Clark. 1995. Nitric oxide produced by activated astrocytes rapidly and reversibly inhibits cellular respiration. *Neurosci. Lett.* 193:201-204.
  141. Bolanos,J.P., A.Almeida, V.Stewart, S.Peuchen, J.M.Land, J.B.Clark, and S.J.Heales. 1997. Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. *J. Neurochem.* 68:2227-2240.
  142. Kapoor,R., M.Davies, P.A.Blaker, S.M.Hall, and K.J.Smith. 2003. Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. *Ann. Neurol.* 53:174-180.
  143. Zielasek,J., H.Reichmann, H.Kunzig, S.Jung, H.P.Hartung, and K.V.Toyka. 1995. Inhibition of brain macrophage/microglial respiratory chain enzyme activity in experimental autoimmune encephalomyelitis of the Lewis rat. *Neurosci. Lett.* 184:129-132.
  144. Mahad,D.J., I.Ziabreva, G.Campbell, N.Lax, K.White, P.S.Hanson, H.Lassmann, and D.M.Turnbull. 2009. Mitochondrial changes within axons in multiple sclerosis. *Brain* 132:1161-1174.
  145. Coleman,M. 2005. Axon degeneration mechanisms: commonality amid diversity. *Nat. Rev. Neurosci.* 6:889-898.
  146. Bechtold,D.A., R.Kapoor, and K.J.Smith. 2004. Axonal protection using flecainide in experimental autoimmune encephalomyelitis. *Ann. Neurol.* 55:607-616.



147. Lo, A.C., C.Y.Saab, J.A.Black, and S.G.Waxman. 2003. Phenytoin protects spinal cord axons and preserves axonal conduction and neurological function in a model of neuroinflammation in vivo. *J. Neurophysiol.* 90:3566-3571.
148. Sospedra, M. and R.Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23:683-747.
149. Link, J., M.Soderstrom, T.Olsson, B.Hojeberg, A.Ljungdahl, and H.Link. 1994. Increased transforming growth factor-beta, interleukin-4, and interferon-gamma in multiple sclerosis. *Ann. Neurol.* 36:379-386.
150. Rieckmann, P., M.Albrecht, B.Kitze, T.Weber, H.Tumani, A.Broocks, W.Luer, and S.Poser. 1994. Cytokine mRNA levels in mononuclear blood cells from patients with multiple sclerosis. *Neurology* 44:1523-1526.
151. Navikas, V. and H.Link. 1996. Review: cytokines and the pathogenesis of multiple sclerosis. *J. Neurosci. Res* 45:322-333.
152. van Boxel-Dezaire, A.H., S.C.Hoff, B.W.van Oosten, C.L.Verweij, A.M.Drager, H.J.Ader, J.C.van Houwelingen, F.Barkhof, C.H.Polman, and L.Nagelkerken. 1999. Decreased interleukin-10 and increased interleukin-12p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. *Ann. Neurol.* 45:695-703.
153. Shamash, S., F.Reichert, and S.Rotshenker. 2002. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J. Neurosci.* 22:3052-3060.
154. Siebert, H. and W.Bruck. 2003. The role of cytokines and adhesion molecules in axon degeneration after peripheral nerve axotomy: a study in different knockout mice. *Brain Res* 960:152-156.
155. Probert, L., K.Akassoglou, G.Kassiotis, M.Pasparakis, L.Alexopoulou, and G.Kollias. 1997. TNF-alpha transgenic and knockout models of CNS inflammation and degeneration. *J. Neuroimmunol.* 72:137-141.
156. Bitsch, A., T.Kuhlmann, Da Costa C., S.Bunkowski, T.Polak, and W.Bruck. 2000. Tumour necrosis factor alpha mRNA expression in early multiple sclerosis lesions: correlation with demyelinating activity and oligodendrocyte pathology. *Glia* 29:366-375.
157. Pender, M.P. and M.J.Rist. 2001. Apoptosis of inflammatory cells in immune control of the nervous system: role of glia. *Glia* 36:137-144.
158. Baker, D., D.Butler, B.J.Scallon, J.K.O'Neill, J.L.Turk, and M.Feldmann. 1994. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur. J. Immunol.* 24:2040-2048.
159. van Oosten, B.W., F.Barkhof, L.Truyen, J.B.Boringa, F.W.Bertelsmann, B.M.von Blomberg, J.N.Woody, H.P.Hartung, and C.H.Polman. 1996. Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* 47:1531-1534.
160. Billiau, A., H.Heremans, F.Vandekerckhove, R.Dijkmans, H.Sobis, E.Meulepas, and H.Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J. Immunol.* 140:1506-1510.
161. Krakowski, M. and T.Owens. 1996. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. *Eur. J. Immunol.* 26:1641-1646.
162. Panitch, H.S., R.L.Hirsch, J.Schindler, and K.P.Johnson. 1987. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. *Neurology* 37:1097-1102.
163. Schonrock, L.M., G.Gawlowski, and W.Bruck. 2000. Interleukin-6 expression in

- human multiple sclerosis lesions. *Neurosci. Lett.* 294:45-48.
164. Smith, T., A. Groom, B. Zhu, and L. Turski. 2000. Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat. Med.* 6:62-66.
  165. Pitt, D., P. Werner, and C.S. Raine. 2000. Glutamate excitotoxicity in a model of multiple sclerosis. *Nat. Med.* 6:67-70.
  166. Matute, C., E. Alberdi, M. Domercq, F. Perez-Cerda, A. Perez-Samartin, and M.V. Sanchez-Gomez. 2001. The link between excitotoxic oligodendroglial death and demyelinating diseases. *Trends Neurosci.* 24:224-230.
  167. Barkhatova, V.P., I.A. Zavalishin, L.S. Askarova, V.K. Shavratskii, and E.G. Demina. 1998. Changes in neurotransmitters in multiple sclerosis. *Neurosci. Behav. Physiol* 28:341-344.
  168. Stover, J.F., U.E. Pleines, M.C. Morganti-Kossmann, T. Kossmann, K. Lowitzsch, and O.S. Kempfski. 1997. Neurotransmitters in cerebrospinal fluid reflect pathological activity. *Eur. J. Clin. Invest* 27:1038-1043.
  169. Sarchielli, P., L. Greco, A. Floridi, A. Floridi, and V. Gallai. 2003. Excitatory amino acids and multiple sclerosis: evidence from cerebrospinal fluid. *Arch. Neurol.* 60:1082-1088.
  170. Werner, P., D. Pitt, and C.S. Raine. 2001. Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann. Neurol.* 50:169-180.
  171. Geurts, J.J., G. Wolswijk, L. Bo, Van der Valk P., C.H. Polman, D. Troost, and E. Aronica. 2003. Altered expression patterns of group I and II metabotropic glutamate receptors in multiple sclerosis. *Brain* 126:1755-1766.
  172. Hardin-Pouzet, H., M. Krakowski, L. Bourbonniere, M. er-Bazes, E. Tran, and T. Owens. 1997. Glutamate metabolism is down-regulated in astrocytes during experimental allergic encephalomyelitis. *Glia* 20:79-85.
  173. Pitt, D., I.E. Nagelmeier, H.C. Wilson, and C.S. Raine. 2003. Glutamate uptake by oligodendrocytes: Implications for excitotoxicity in multiple sclerosis. *Neurology* 61:1113-1120.
  174. Vercellino, M., A. Merola, C. Piacentino, B. Votta, E. Capello, G.L. Mancardi, R. Mutani, M.T. Giordana, and P. Cavalla. 2007. Altered glutamate reuptake in relapsing-remitting and secondary progressive multiple sclerosis cortex: correlation with microglia infiltration, demyelination, and neuronal and synaptic damage. *J. Neuropathol. Exp. Neurol.* 66:732-739.
  175. Newcombe, J., A. Uddin, R. Dove, B. Patel, L. Turski, Y. Nishizawa, and T. Smith. 2008. Glutamate receptor expression in multiple sclerosis lesions. *Brain Pathol.* 18:52-61.
  176. Chao, C.C., S. Hu, L. Ehrlich, and P.K. Peterson. 1995. Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors. *Brain Behav. Immun.* 9:355-365.
  177. van Horssen J., C.M. Vos, L. Admiraal, E. S. Van Haastert, L. Montagne, Van der Valk P., and H.E. de Vries. 2006. Matrix metalloproteinase-19 is highly expressed in active multiple sclerosis lesions. *Neuropathol. Appl. Neurobiol.* 32:585-593.
  178. Newman, T.A., S.T. Woolley, P.M. Hughes, N.R. Sibson, D.C. Anthony, and V.H. Perry. 2001. T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. *Brain* 124:2203-2214.
  179. Lindberg, R.L., C.J. De Groot, L. Montagne, P. Freitag, Van der Valk P., L. Kappos, and D. Leppert. 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.
  180. Gveric, D., R. Hanemaaijer, J. Newcombe, N.A. van Lent, C.F. Sier, and M.L. Cuzner.

2001. Plasminogen activators in multiple sclerosis lesions: implications for the inflammatory response and axonal damage. *Brain* 124:1978-1988.
181. Anthony, D.C., B.Ferguson, M.K.Matzzak, K.M.Miller, M.M.Esiri, and V.H.Perry. 1997. Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. *Neuropathol. Appl. Neurobiol.* 23:406-415.
182. Chandler, S., R.Coates, A.Gearing, J.Lury, G.Wells, and E.Bone. 1995. Matrix metalloproteinases degrade myelin basic protein. *Neurosci. Lett.* 201:223-226.
183. Busch, S.A., K.P.Horn, D.J.Silver, and J.Silver. 2009. Overcoming macrophage-mediated axonal dieback following CNS injury. *J. Neurosci.* 29:9967-9976.
184. Lu, W., M.Bhasin, and S.E.Tsirka. 2002. Involvement of tissue plasminogen activator in onset and effector phases of experimental allergic encephalomyelitis. *J. Neurosci.* 22:10781-10789.
185. Cuzner, M.L., D.Gveric, C.Strand, A.J.Loughlin, L.Paemen, G.Opendakker, and J.Newcombe. 1996. The expression of tissue-type plasminogen activator, matrix metalloproteinases and endogenous inhibitors in the central nervous system in multiple sclerosis: comparison of stages in lesion evolution. *J. Neuropathol. Exp. Neurol.* 55:1194-1204.
186. Teesalu, T., A.E.Hinkkanen, and A.Vaheri. 2001. Coordinated induction of extracellular proteolysis systems during experimental autoimmune encephalomyelitis in mice. *Am. J. Pathol.* 159:2227-2237.
187. Reijerkerk, A., G.Kooij, S.M.van der Pol, T.Leyen, van het Hof B., P.O.Couraud, D.Vivien, C.D.Dijkstra, and H.E.de Vries. 2008. Tissue-type plasminogen activator is a regulator of monocyte diapedesis through the brain endothelial barrier. *J. Immunol.* 181:3567-3574.
188. Flavin, M.P., G.Zhao, and L.T.Ho. 2000. Microglial tissue plasminogen activator (tPA) triggers neuronal apoptosis in vitro. *Glia* 29:347-354.
189. Yin, Y., M.T.Henzl, B.Lorber, T.Nakazawa, T.T.Thomas, F.Jiang, R.Langer, and L.I.Benowitz. 2006. Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nat. Neurosci.* 9:843-852.
190. Liu, X., E.Hawkes, T.Ishimaru, T.Tran, and D.W.Sretavan. 2006. EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. *J. Neurosci.* 26:3087-3101.
191. Rapalino, O., O.Lazarov-Spiegler, E.Agranov, G.J.Velan, E.Yoles, M.Fraidakis, A.Solomon, R.Gepstein, A.Katz, M.Belkin, M.Hadani, and M.Schwartz. 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nat. Med.* 4:814-821.
192. Barrette, B., M.A.Hebert, M.Filali, K.Lafortune, N.Vallieres, G.Gowing, J.P.Julien, and S.Lacroix. 2008. Requirement of myeloid cells for axon regeneration. *J. Neurosci.* 28:9363-9376.
193. Shechter, R., A.London, C.Varol, C.Raposo, M.Cusimano, G.Yovel, A.Rolls, M.Mack, S.Pluchino, G.Martino, S.Jung, and M.Schwartz. 2009. Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS. Med.* 6:e1000113.
194. Kigerl, K.A., J.C.Gensel, D.P.Ankeny, J.K.Alexander, D.J.Donnely, and P.G.Popovich. 2009. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J. Neurosci.* 29:13435-13444.
195. Kotter, M.R., A.Setzu, F.J.Sim, van Rooijen N., and R.J.Franklin. 2001. Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. *Glia* 35:204-212.
196. Kotter, M.R., C.Zhao, van Rooijen N., and R.J.Franklin. 2005. Macrophage-

- depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. *Neurobiol. Dis.* 18:166-175.
197. Loughlin, A.J., C.A. Copelman, A. Hall, T. Armer, B.C. Young, D.N. Landon, and M.L. Cuzner. 1997. Myelination and remyelination of aggregate rat brain cell cultures enriched with macrophages. *J. Neurosci. Res.* 47:384-392.
  198. Kerschensteiner, M., E. Gallmeier, L. Behrens, V.V. Leal, T. Misgeld, W.E. Klinkert, R. Kolbeck, E. Hoppe, R.L. Oropeza-Wekerle, I. Bartke, C. Stadelmann, H. Lassmann, H. Wekerle, and R. Hohlfeld. 1999. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J. Exp. Med.* 189:865-870.
  199. Kerschensteiner, M., C. Stadelmann, G. Dechant, H. Wekerle, and R. Hohlfeld. 2003. Neurotrophic cross-talk between the nervous and immune systems: implications for neurological diseases. *Ann. Neurol.* 53:292-304.
  200. Stadelmann, C., M. Kerschensteiner, T. Misgeld, W. Bruck, R. Hohlfeld, and H. Lassmann. 2002. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain* 125:75-85.
  201. Boven, L.A., Van Meurs M., Van Zwam M., A. Wierenga-Wolf, R.Q. Hintzen, R.G. Boot, J.M. Aerts, S. Amor, E.E. Nieuwenhuis, and J.D. Laman. 2006. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* 129:517-526.
  202. Fabriek, B.O., E.S. Van Haastert, I. Galea, M.M. Polfliet, E.D. Dopp, M.M. Van Den Heuvel, T.K. Van Den Berg, C.J. De Groot, Van der Valk P., and C.D. Dijkstra. 2005. CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia* 51:297-305.
  203. Teunissen, C.E., C.D. Dijkstra, B. Jasperse, F. Barkhof, H. Vanderstichele, E. Vanmechelen, C.H. Polman, and L. Bo. 2006. Growth-associated protein 43 in lesions and cerebrospinal fluid in multiple sclerosis. *Neuropathol. Appl. Neurobiol.* 32:318-331.
  204. Doyle, A.G., G. Herbein, L.J. Montaner, A.J. Minty, D. Caput, P. Ferrara, and S. Gordon. 1994. Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur. J. Immunol.* 24:1441-1445.
  205. Conrad, D.J., H. Kuhn, M. Mulkins, E. Highland, and E. Sigal. 1992. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A* 89:217-221.
  206. Buechler, C., M. Ritter, E. Orso, T. Langmann, J. Klucken, and G. Schmitz. 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and anti-inflammatory stimuli. *J. Leukoc. Biol.* 67:97-103.
  207. Willment, J.A., H.H. Lin, D.M. Reid, P.R. Taylor, D.L. Williams, S.Y. Wong, S. Gordon, and G.D. Brown. 2003. Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J. Immunol.* 171:4569-4573.
  208. Raes, G., L. Brys, B.K. Dahal, J. Brandt, J. Grooten, F. Brombacher, G. Vanham, W. Noel, P. Bogaert, T. Boonefaes, A. Kindt, Van den Bergh R., P.J. Leenen, De Baetselier P., and G.H. Ghassabeh. 2005. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. *J. Leukoc. Biol.* 77:321-327.
  209. te Velde, A.A., R.J. Huijbens, J.E. De Vries, and C.G. Figdor. 1990. IL-4 decreases Fc gamma R membrane expression and Fc gamma R-mediated cytotoxic activity of

- human monocytes. *J. Immunol.* 144:3046-3051.
210. Van Ginderachter, J.A., K.Movahedi, G.G.Hassanzadeh, S.Meerschaut, A.Beschin, G.Raes, and De Baetselier P. 2006. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* 211:487-501.
  211. Torocsik, D., H.Bardos, L.Nagy, and R.Adany. 2005. Identification of factor XIII-A as a marker of alternative macrophage activation. *Cell Mol. Life Sci.* 62:2132-2139.
  212. Martinez, F.O., S.Gordon, M.Locati, and A.Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* 177:7303-7311.
  213. Skeen, M.J., M.A.Miller, T.M.Shinnick, and H.K.Ziegler. 1996. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. *J. Immunol.* 156:1196-1206.
  214. Gerber, J.S. and D.M.Mosser. 2001. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J. Immunol.* 166:6861-6868.
  215. Anderson, C.F. and D.M.Mosser. 2002. A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J. Leukoc. Biol.* 72:101-106.
  216. Verreck, F.A., de Boer T., D.M.Langenberg, M.A.Hoeve, M.Kramer, E.Vaisberg, R.Kastelein, A.Kolk, R.de Waal-Malefyt, and T.H.Ottenhoff. 2004. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc. Natl. Acad. Sci. U. S. A* 101:4560-4565.
  217. Hart, P.H., G.F.Vitti, D.R.Burgess, G.A.Whitty, D.S.Piccoli, and J.A.Hamilton. 1989. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc. Natl. Acad. Sci. U. S. A* 86:3803-3807.
  218. Kodolja, V., C.Muller, O.Politz, N.Hakij, C.E.Orfanos, and S.Goerd. 1998. Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *J. Immunol.* 160:1411-1418.
  219. Bonecchi, R., S.Sozzani, J.T.Stine, W.Luini, G.D'Amico, P.Allavena, D.Chantry, and A.Mantovani. 1998. Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* 92:2668-2671.
  220. Imai, T., M.Nagira, S.Takagi, M.Kakizaki, M.Nishimura, J.Wang, P.W.Gray, K.Matsushima, and O.Yoshie. 1999. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* 11:81-88.
  221. Liddiard, K., J.S.Welch, J.Lozaich, S.Heinz, C.K.Glass, and D.R.Greaves. 2006. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. *BMC. Mol. Biol.* 7:45.
  222. Raes, G., De Baetselier P., W.Noel, A.Beschin, F.Brombacher, and G.G.Hassanzadeh. 2002. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J. Leukoc. Biol.* 71:597-602.