

University of Groningen

## Translational multiple sclerosis research in primates

Dunham, Jordon Tyler-Nathan

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2017

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Dunham, J. T-N. (2017). *Translational multiple sclerosis research in primates: Mind the gap*. Rijksuniversiteit Groningen.

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

# A qualitative and quantitative analysis of grey matter demyelination in different experimental autoimmune encephalomyelitis induction protocols in marmosets

Jordon Dunham<sup>1,2</sup>, Hans Lassman<sup>3</sup>, Patrick Peschl<sup>4</sup>, Markus Reindl<sup>4</sup>,  
Nikki van Driel<sup>1</sup>, Jon D. Laman<sup>2</sup>, Bert A. 't Hart<sup>1,2</sup>, Jan Bauer<sup>3</sup> and  
Yolanda S. Kap<sup>1</sup>

<sup>1</sup>Dept. of Immunobiology, Biomedical Primate Research Centre, Rijswijk, The Netherlands;

<sup>2</sup>University of Groningen, University Medical Center, Dept. of Neuroscience, section Medical Physiology Groningen, The Netherlands;

<sup>3</sup>Dept. Neuroimmunology, Brain Research Institute, Medical University, Vienna, Austria;

<sup>4</sup>Clinical Dept. of Neurology, Innsbruck Medical University, Innsbruck, Austria.

JB and YSK share senior authorship.

## Abstract

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterized by myelin loss and axonal damage in both the white matter (WM) and grey matter (GM). Despite being traditionally viewed as a WM disease, GM demyelination and atrophy are key characteristics of the MS brain and are positively correlated with motor and cognitive disability. In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), GM pathology varies per species and is only present in some Lewis rat strains and in the Neotropical primate common marmoset (*Callithrix jacchus*). In this study, we performed a comparative analysis of GM pathology between different marmoset EAE induction protocols, with the aim to gain insight into the underlying pathogenic mechanism(s). We observed the highest incidence of animals with GM lesions and the highest percentage of GM demyelination after immunization with human myelin emulsified in complete Freund's adjuvant (CFA). Monkeys inoculated with MOG peptide 34 to 56 (MOG34-56) emulsified in either CFA or IFA, displayed clear GM demyelination although no pathogenic anti-MOG antibodies were found, suggesting a role for T lymphocytes or a soluble factor in GM lesion development. The higher extent of GM demyelination in models induced with CFA suggests a contribution of myeloid cells activated by factors present in CFA, but not in IFA. Future work to define the rate-limiting mechanism(s) driving GM injury, particularly in monkeys with aggressive pathology, and refinement of EAE induction protocol are warranted.



## Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory/demyelinating disease of the human central nervous system (CNS) that affects an estimated 2.3 million people globally <sup>1</sup>. The prominent pathological feature of MS is multi-focal demyelinated lesions (plaques) that are variably associated with a combined cellular and humoral autoimmune attack on myelin <sup>2</sup>. Lesions in the MS brain are disseminated through both the white matter (WM) and grey matter (GM) and while historically MS was viewed predominantly as a WM disease, it is now increasingly clear that the extent of focal WM damage alone cannot account for all symptoms observed in patients. Both physical disability and cognitive deficits are more strongly correlated with GM injury than with WM T1 hypointense and T2 hyperintense lesion load <sup>3,4-6</sup>.

Mechanistically it is unclear whether the GM injury observed in the MS brain results from a primary (e.g. neuronal susceptibility or oligodendrocyte defects) or secondary pathogenic process (e.g. virtual hypoxia, pathogenic antibodies) <sup>3</sup>. Injury to the GM occurs in all layers of the cortex and of the three patterns of cortical demyelination, subpial lesions are the most abundant <sup>7</sup>. The predilection for subpial demyelination to occur in deep sulci regions of the cortex has led some to hypothesize that these lesion types may be mediated by factors present in the cerebrospinal fluid (CSF) such as cytokines or heat shock proteins; however, as to date, no single factor has been isolated to explain this phenomenon <sup>8</sup>.

Injury to the GM is variably reflected in rodent models of MS. The frequently used myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) model in C57BL/6 mice has a strong predilection for spinal cord WM pathology, yet notoriously lacks GM injury in the brain <sup>9</sup>. In comparison, demyelination of the cerebral cortex can be observed in the Lewis rat EAE model when using LEW.AR1 (RT1<sup>R2</sup>) and LEW.1W (RT1<sup>U</sup>) strains sensitized against rhMOG and in Biozzi antibody high (AB/H) mice when immunized with neurofilament proteins <sup>10-12</sup>. Furthermore, stereotactic injection of cytokines into the cerebral cortex or subarachnoid space of MOG-immunized rats (Dark Agouti and Lewis) manages to replicate human cortical MS lesions <sup>10,13</sup>.

Induction of EAE in the common marmoset (*Callithrix jacchus*) results in the development of MS-like GM pathology <sup>14-16</sup>. GM pathology in the marmoset model occurs in all layers of the cortex and mirrors patterns of intracortical, leukocortical and subpial demyelination found in the MS brain <sup>17</sup>. Thinning of the cortex has also been observed in marmosets immunized with rhMOG emulsified in complete Freund's adjuvant (CFA) followed by an injection of inactivated *Bordetella pertussis* suggesting that these monkeys exhibit neurodegenerative aspects of MS <sup>10,16,17</sup>.

A major focus of marmoset EAE research at the Biomedical Primate Research Centre (BPRC; Rijswijk, NL) over the last two decades has been refinement of the model. These efforts have resulted in substitution of CFA for incomplete Freund's adjuvant (IFA) and the use of a single peptide instead of MOG protein in EAE induction protocols. It is unclear if this has had an impact on the development of GM injury. We hypothesized that the innate stimulation by mycobacterial components in CFA influences GM injury. Collectively, the results presented in this chapter show that the development of GM lesions varies considerably per individual, even within the same induction protocol, and that exclusion of mycobacterial component in immunization method had deleterious effects on GM pathology development.

## Material and Methods

### Marmoset EAE tissue

Archival formalin-fixed, paraffin-embedded tissues and cryogenically preserved plasma samples derived from prior EAE experiments conducted at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands) were used for this study; hence no animal was directly sacrificed for this research. Marmosets in studies were obtained from the purpose-bred colony at the BPRC and all studies were performed under the approval of the institutional ethics review committee in accordance to Dutch law.

To compare the effect of immunization on pathology development, we examined marmoset EAE tissue (**Table 1**) from non-treated monkeys inoculated with: human myelin emulsified in CFA, myelin from MOG-deficient C57BL/6 mice (MOG<sup>-/-</sup> myelin) in CFA, unglycosylated human recombinant myelin oligodendrocyte glycoprotein (rhMOG1-125) in CFA or IFA, and synthetic peptide representing amino acids 34-56 of human MOG (MOG34-56) in CFA or IFA. Hence, all tested immunization methods involved in the refinement of the marmoset EAE model are represented in this study.

### EAE induction

A detailed description of disease induction and monitoring can be found elsewhere<sup>18</sup>. Briefly, induction of EAE was performed by injection of antigen-adjuvant emulsion into the dorsal skin of sedated marmosets. During experiments, marmosets were monitored daily for the development of neurological symptoms. A standardized scoring system was utilized for daily monitoring of overt clinical signs whereby score 2 = ataxia or optic disease; and 2.5 = paresis of two limbs. Humane euthanasia for ethical reasons was performed once monkeys exhibited paresis of two or more limbs (score 2.5) or paralysis of one or more limbs (score 3). The monkeys for this study were selected based upon the development of clinically evident disease severe enough to warrant humane euthanasia, unless otherwise noted.

### Pathological characterization by immunohistochemistry

Immunohistochemical analysis of formalin-fixed paraffin embedded material was performed on 5 µm sections. Sections were deparaffinized using xylene (VWR, Radnor, PA) and then rehydrated via graded ethanol into distilled water. To block endogenous peroxidase activity, tissue sections were incubated in 0.03% hydrogen peroxide (Sigma, St. Louis, MO) in methanol for 30 min and heat-induced antigen retrieval (steaming) was performed in either EDTA (pH 8.6; Sigma) or citrate (pH 6.0; Sigma). After antigen retrieval, tissue was rinsed twice in Tris-buffered saline (TBS). Tissue sections were incubated in 10% FCS in Dako wash buffer (DAKO, Glostrup, Denmark) for 30 min to block non-specific antibody binding.

Tissue sections were incubated with primary antibodies overnight at 4°C and several wash steps (TBS) were performed to remove non-specific Ab binding. Visualization of bound antibody was performed using the avidin-biotin complex (ABC) method. Biotin-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were added to tissue sections for 1 h at room temperature and sections were subjected to additional wash steps. Detection of ABC binding was done with avidin-labeled peroxidase (Sigma, 1:150) and



Table 1. Animals used in this study

Animal	Gender	Age (months) <sup>a</sup>	Immunization	Day of sacrifice	Max. EAE score
1	F	33	Myelin/CFA	Week 7	2
2	F	N.A.	Myelin/CFA	21	3
3	M	45	Myelin/CFA	Week 35	2
4	M	39	Myelin/CFA	46	N.A. <sup>b</sup>
5	M	39	Myelin/CFA	46	N.A. <sup>b</sup>
6	M	57	Myelin/CFA	Week 76	1
7	M	24	rhMOG/CFA	111	2.5
8	M	42	rhMOG/CFA	55	2.5
9	M	64	rhMOG/CFA	57	3
10	M	128	rhMOG/CFA	55	3
11	M	86	rhMOG/CFA	59	2.5
12	M	33	rhMOG/CFA	55	3
13	M	27	rhMOG/CFA	83	3
14	M	27	rhMOG/CFA	91	3
15	M	27	rhMOG/CFA	105	0.5
16	M	28	rhMOG/IFA	33	2.5
17	M	50	rhMOG/IFA	26	2.5
18	M	33	rhMOG/IFA	28	2.5
19	F	30	rhMOG/IFA	65	3
20	F	35	rhMOG/IFA	57	3
21	F	39	rhMOG/IFA	81	3
22	F	39	MOG34-56/CFA	71	3
23	F	24	MOG34-56/CFA	78	3
24	F	34	MOG34-56/CFA	86	2.5
25	F	30	MOG34-56/CFA	61	3
26	F	59	MOG34-56/IFA	62	2
27	F	50	MOG34-56/IFA	85	2.5
28	F	47	MOG34-56/IFA	106	2.5
29	M	68	MOG34-56/IFA	85	2.5
30	M	38	MOG34-56/IFA	87	2.5
31	M	26	MOG34-56/IFA	51	3
32	F	29	MOG34-56/IFA	154	0
33	F	34	MOG34-56/IFA	60	2.5

<sup>a</sup> Age at the end of the study.<sup>b</sup> N.A. not available

diaminobenzidine tetrachloride (DAB, Sigma). A hemalaun counterstain was performed to visualize nuclei. Finally tissue sections were dehydrated with graded ethanol and xylene and then mounted with malinol (Waldeck, Münster, Germany).

### Quantification of demyelination

To select tissue for comparison of immunization methods, we first performed a pathological characterization of non-treated EAE marmosets that developed neurological symptoms to assess the presence of GM pathology. For each marmoset (n=111), 4-12 coronally cut areas from the front, mid and hindbrain were analyzed. A binary (yes/no) screening was performed for the presence of leukocortical, intracortical and subpial lesions. From the group of marmosets that had developed clinically evident EAE and were GM+, a selection was made for further characterization (see **Table 1**). Each immunization group, with the exception of MOG34-56/CFA immunized marmosets, contained a minimum of 6 monkeys. Lower numbers in the MOG34-56/CFA are attributed to tissue availability and reflect a limited usage of this model.

To determine the area of demyelination of GM and WM, marmoset tissue sections were stained for PLP as described above. Tissue slides were scanned at high resolution (2000 dpi) and images were analyzed using Nanozoomer Digital Pathology software. Standard scale bars were used to calculate area of demyelination (mm<sup>2</sup>) and data were then expressed as a percentage of the whole WM or GM.

### MOG-IgG immunofluorescence assays

The presence of pathogenic antibodies to conformationally intact MOG in marmoset EAE sera was determined by a well-characterized cell surface live staining immunofluorescence technique as previously reported<sup>19,20</sup>. Briefly, HEK-293A cells were transfected with human MOG cloned into mammalian expression vector (Vivid Colours™ pcDNA™ 6.2-EmGFP/TOPO; Invitrogen) resulting in the expression of MOG fused C-terminally to EmGFP. A Cy3-conjugated goat anti-human IgG antibody was used for detection of marmoset serum MOG-IgG on the surface of the MOG expressing cells. Cells were incubated with marmoset immune plasma samples (diluted 1:20 and 1:40) for 1 h at 4° C following a blocking step with goat IgG (Sigma-Aldrich). Human serum probes that are known anti-MOG IgG positive and negative were used as positive and negative controls in each assay as a quality control. A seropositive cutoff of 1:160 dilution was applied based on previous findings in human MS serum<sup>20</sup>.

### Figures and statistics

Figures were compiled with Adobe InDesign CC 2015 (Adobe Systems, San Jose, CA) and then adjusted with Adobe Photoshop CC 2015 (Adobe Systems). Statistical analysis (Mann-Whitney test and Spearman correlation) was performed using Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).



## Results

### A pathological characterization

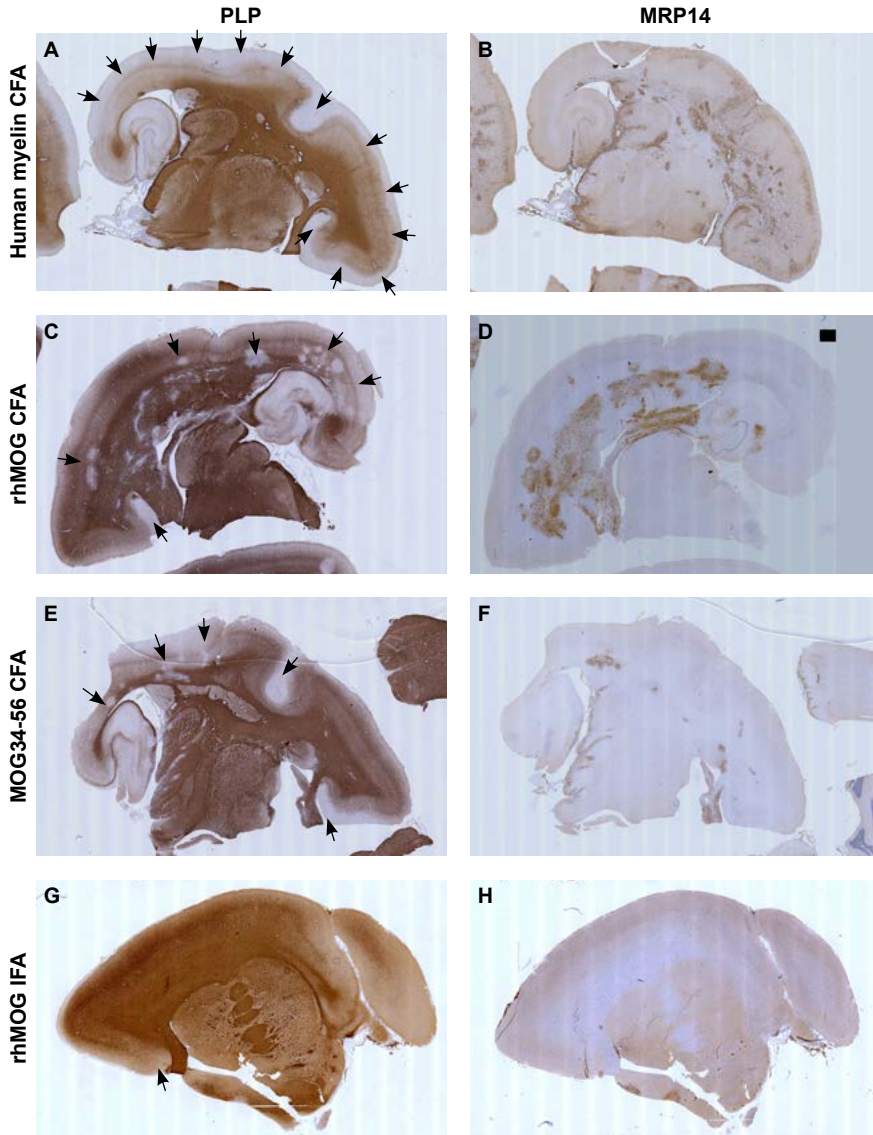
The first EAE induction protocol in the marmoset EAE model used a homogenate isolated from the WM of an MS patient emulsified in commercial CFA, containing *Mycobacterium tuberculosis* for stimulation of innate immunity<sup>21</sup>. In these animals, widespread demyelination of the GM was frequently observed (**Figure 1A-B**). When present, subpial demyelination was often extensive (**Figure 1A**, arrow), and in some cases, injury extended into all layers of the cortex and spanned large sections of the sulci and gyri (**Figure 1A**). Leukocortical and intracortical lesions in this model were routinely observed and like subpial demyelination, considerable heterogeneity of the extent and stage of activity of lesions were noted. To reduce the time required for development of clinically evident disease, new EAE induction protocols were established, which were based on immunization with rhMOG or MOG34-56 emulsified in CFA. As shown in **Figure 1C-F**, inflammation and demyelination in both WM and GM were routinely observed in these models. Whereas both protocols elicited robust GM injury, demyelination was frequently noted as less extensive than in human myelin (CFA) immunized monkeys. For subpial demyelination, the sulci and areas adjacent to demyelinating WM corpus callosum regions were noted as predilection sites for lesion development. Despite predilection areas of GM subpial injury, like in human myelin/CFA immunized monkeys, demyelination was observed in gyri of the cortex independent of demyelination of the sulci. Similarly, leukocortical and intracortical lesions were frequently found in both models and were also heterogeneous in size and activity.

A key step in the model refinement process was the elimination of the bacterial component from CFA via immunization with antigen emulsified in the more animal welfare friendly IFA. In monkeys that were immunized with rhMOG emulsified in IFA, GM demyelination could be detected in some monkeys. However, whereas all models exhibited considerable heterogeneity between marmosets with respect to the extent of GM demyelination, and thus minor amounts could be observed in some monkeys of the CFA models, GM demyelination was noticeably less in the rhMOG/IFA monkeys; both in relation to the number of monkeys that developed GM demyelination and to the extent of the GM injury.

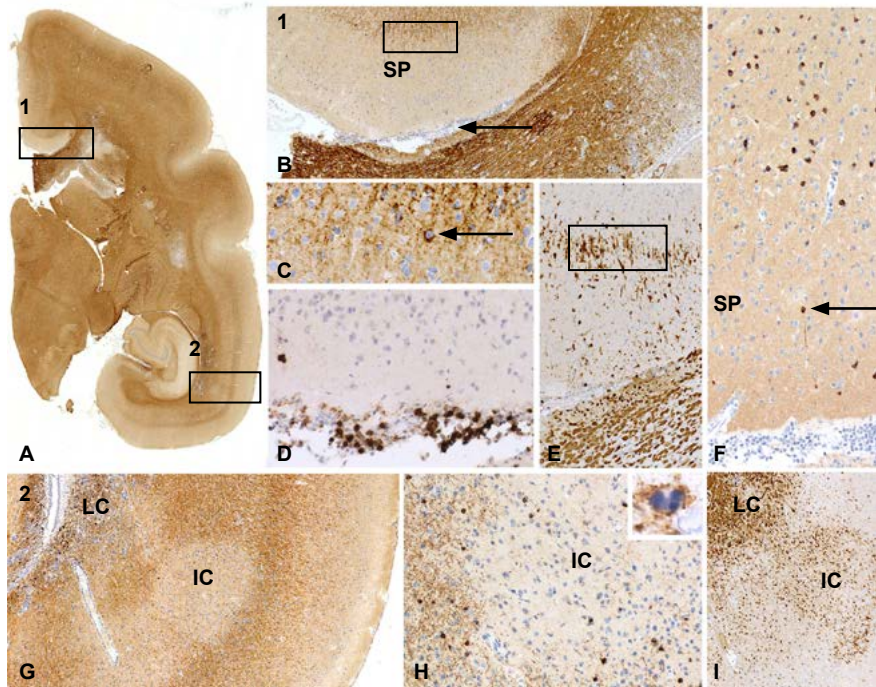
### A pathological characterization of the most refined model

Immunization with the single encephalitogenic peptide MOG34-56 in IFA represents the minimal requirement to reproducibly induce MS-like pathology and disease. As shown in **Figure 2A**, this refined protocol also elicits subpial, intracortical and leukocortical demyelination (**Figure 2A, Box 1-2**). Whereas GM and WM demyelination varied between individual marmosets, extensive demyelination of both regions could be observed. Leukocortical lesions often were associated with large demyelinating WM lesions and generally were paucicellular compared to the adjacent WM area (**Figure 2G**). Intracortical regions were found adjacent to leukocortical and subpial lesions, but could also be observed independent of other lesion types. Subpial lesions were frequently observed near inflamed corpus callosum regions, often associated with macrophage/microglia activation and could penetrate deep into the cortex (**Figure 2**). Like in all other models examined, demyelination was associated with variable loss of oligodendrocytes





**Figure 1. Pathological characterization of different EAE models.** Brains from marmosets with EAE induced by different immunization protocols were pathologically characterized for demyelination (proteolipid protein (PLP)) and inflammation (MRP14) in white matter (WM) and grey matter (GM). The depicted brain slices are from marmosets with EAE induced with: human myelin emulsified in CFA (A-B), human recombinant myelin oligodendrocyte glycoprotein (rhMOG) in CFA (C-D), synthetic peptide representing amino acids 34-56 of human MOG (MOG34-56) in CFA (E-F) and rhMOG in IFA (G-H). Arrows indicate areas of demyelination.

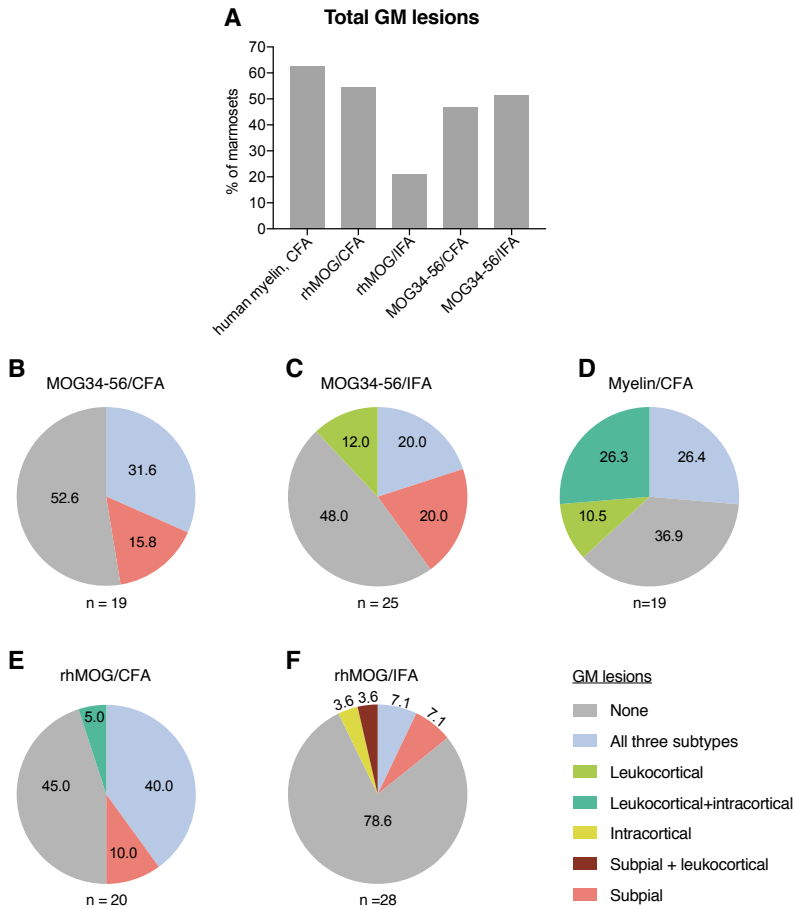


**Figure 2. A refined model: MOG34-56/IFA.** Immunization with the encephalitogenic peptide MOG34-56 emulsified in IFA is the minimal requirement to induce clinical EAE. Shown in **A** is a PLP staining highlighting areas of subpial (box 1), leukocortical and intracortical lesions (box 2). Box 1 is magnified in **B-F**. Subpial (SP) lesions were frequently found adjacent to active white matter corpus callosum lesions (**B**; arrow = lesion). The border of these lesions could extend deep into the grey matter, often containing PLP positive macrophages (arrow in **C**), meningeal infiltrates including T cells (**D**) and expression of MRP14 in microglia (box in **E**). Demyelinated subpial areas contained fewer oligodendrocytes (**F**; arrow points at a residual oligodendrocyte) compared to non-demyelinated areas (top of **F**). Box 2 is magnified in **G** showing an intracortical (IC) and a leukocortical (LC) lesion. **H**, CNPase staining showing myelinating oligodendrocytes at the edge of the intracortical (IC) lesion with lesion center relatively devoid of oligodendrocytes (Inset = an apoptotic oligodendrocyte). **I**, detection of MRP14 expression was observed in these lesions.

depending on lesion activity and stage (**Figure 2F**). Thus, even with elimination of bacterial components from adjuvant and immunization with a single peptide sequence, MS-like GM pathology could be elicited.

#### A quantitative comparison of models

Following a gross pathological assessment, we performed a comparative quantitative analysis of the ability of the different EAE induction protocols to elicit leukocortical, intracortical and subpial demyelination. Shown in **Figure 3A** is the percentage of marmosets per EAE induction protocol that developed GM injury. The model with the least number of monkeys with GM injury was the rhMOG/IFA model (21%). In contrast, 55 and 63% of rhMOG/CFA and human myelin/CFA

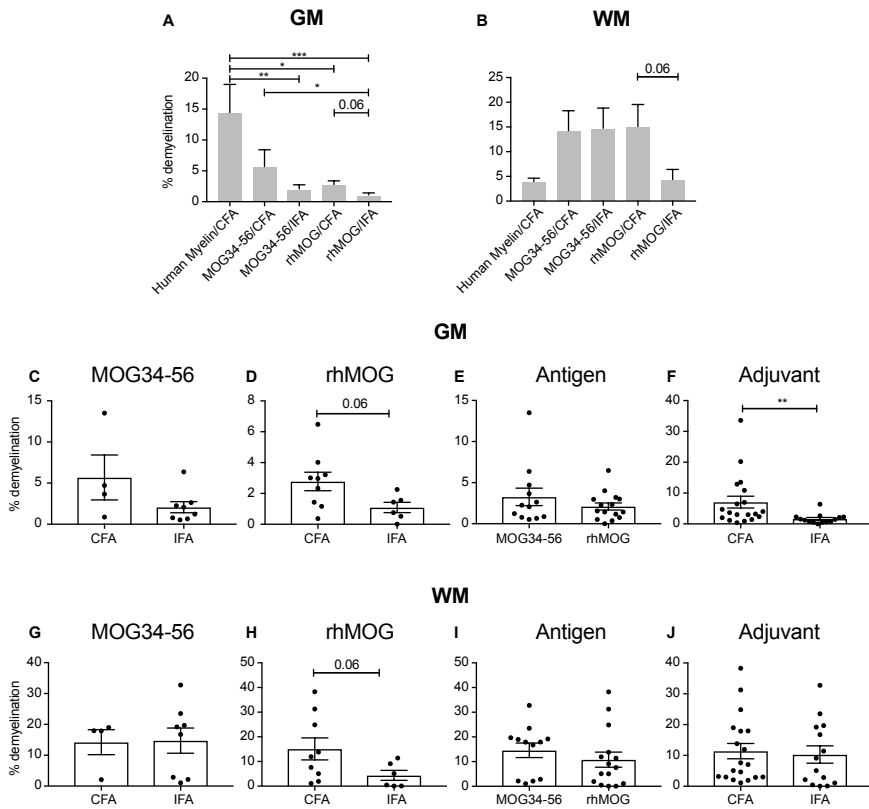


**Figure 3. GM lesion assessment in the different induction protocols.** Marmoset EAE brains were assessed for grey matter (GM) lesions. **A** shows the percentage of marmosets per EAE induction method that developed GM lesions. Shown in **B-F** are pie charts depicting the percentage of GM lesion types observed per EAE induction protocol (color codes explained in legend).

immunized monkeys, respectively, were positive for GM lesions. Monkeys immunized with MOG34-56 emulsified in CFA (47%) or IFA (52%) were intermediate in development of GM pathology. Shown in **Figure 3B-F** are the percentages of animals with each type of GM lesion observed. Whereas few monkeys developed intracortical lesions only, monkeys frequently developed either all three types of lesions or subpial lesions alone. In both human myelin/CFA and MOG34-56/IFA immunized marmosets, a subgroup of monkeys developed leukocortical lesions only (10 and 12%, respectively).



We next quantified the extent of both brain WM and GM demyelination as a percentage of the respective areas (**Figure 4**). GM demyelination was the lowest in the rhMOG/IFA ( $\bar{x} = 1.1\%$ ) immunized marmosets and highest in the human myelin/CFA ( $\bar{x} = 14.4\%$ ) immunized monkeys. Notably, both of these groups displayed the least WM demyelination (**Figure 4A-B**). With respect to GM injury, the percentage of demyelination was significantly higher ( $p < 0.05$ ) in the human myelin/CFA marmosets compared to all groups except for MOG34-56/CFA monkeys (**Figure 4A**). Although the mean percentage of demyelination was 5.7% in MOG34-56/CFA marmosets and only 2.1% in the MOG34-56/IFA monkeys, no significant difference was found between these two groups (**Figure 4C**). When comparing rhMOG/CFA and rhMOG/IFA immunized animals for WM and GM demyelination, the IFA group trended towards being significantly lower ( $p = 0.06$ ) than

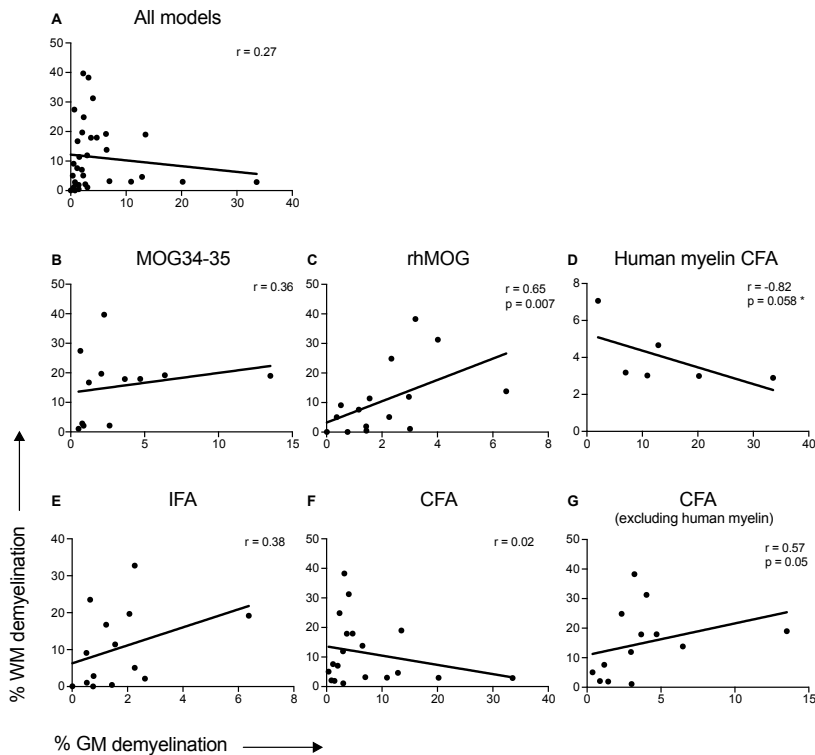


**Figure 4. Quantitative comparison of GM and WM demyelination different the different protocols.** White matter (WM) and grey matter (GM) demyelination were quantified by analyzing PLP staining of coronally cut areas (4-12/marmoset) from the front, mid and hindbrain. The areas of demyelination were then expressed as a percentage of total WM or GM surface area analyzed. Shown are a comparison of WM and GM demyelination of all induction protocols (**A-B**), by antigen in relation to adjuvant (**C-D, G-H**), differences between antigens regardless of adjuvant (**E,I**) and differences between adjuvants regardless of antigen (**F,J**). Note that the positive significant impact of CFA on GM demyelination area was also observed when excluding marmosets immunized with human myelin (data not shown).

the CFA-immunized marmosets (**Figure 4D, H**). The overall amount of GM demyelination was significantly lower ( $p < 0.05$ ) in IFA-based induction protocols when comparing adjuvant irrespective of immunization antigen, but not when comparing antigen irrespective of adjuvant used; no trends with respect to WM were observed (**Figure 4E-F, I-J**). These differences between CFA and IFA induction protocols were also observed when human myelin/CFA-immunized marmosets were excluded from the analysis ( $p = 0.02$ ; data not shown).

**The relationship between WM and GM demyelination**

In the marmosets that developed GM lesions, we next determined to which extent GM demyelination correlated to WM demyelination. Shown in **Figure 5** is the percentage WM demyelination plotted against the percentage GM demyelination for all marmosets analyzed (**A**), per immunizing antigen (**B-D**) and by adjuvant (**E-G**). Whereas a positive significant correlation ( $r = 0.65$ ,  $p = 0.007$ ) could be observed for the rhMOG-immunized monkeys, WM demyelination



**Figure 5. Correlation between WM and GM demyelination.** Shown is the correlation between the percentage of demyelination of white matter (WM) and grey matter (GM) for all EAE induction protocols (**A**), individual antigen (**B-D**) and adjuvant (**E-G**). No correlation was observed when the outlier was excluded (**D**).



was not significantly correlated to GM injury in the MOG34-56 group (**Figure 5B-C**). For the human myelin-immunized monkeys (**Figure 5D**), there was a negative correlation trend, although no significance was observed; the correlation was not observed when eliminating an outlier from the data set (data not shown). With respect to adjuvant, no significant correlations between WM and GM demyelination were observed in either IFA or CFA immunized monkeys (**Figure 5E-F**). However, when excluding marmosets from the human myelin immunized group, WM demyelination positively ( $r = 0.57$ ;  $p = 0.05$ ) correlated with GM demyelination over all remaining groups (**Figure 5G**). Thus, the degree of correlation between WM and GM varies per disease induction protocol.

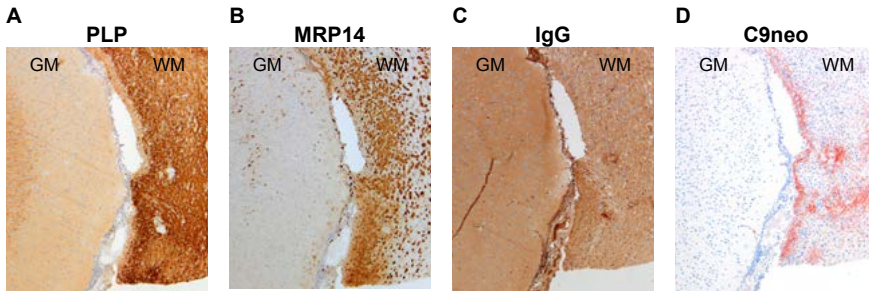
### The role of auto-antibodies in demyelination

As all marmoset EAE induction protocols make use of active immunization with self-antigen, pathogenic auto-antibodies may be induced and play a role in disease progression and GM injury. Hence, we assessed the presence of IgG and C9neo in EAE brain tissue. Marmosets that were immunized with myelin from MOG KO and WT mice were included in this analysis as controls. In all models, IgG deposition was widespread in both WM and GM (**Figure 6A-C**). In contrast, complement activation (C9neo deposition) was observed only in early active lesions, characterized by macrophages/microglia with intracellular MOG (**Figure 6A-B, D**). In the early active, demyelinating areas, deposition of C9neo was readily discernible in WM, and in leukocortical and intracortical lesions (**Figure 6D**). As the uptake of IgG and C9neo by macrophages/microglia was not as easily discernible as uptake of PLP, sera were tested for the ability to bind to conformational MOG. Regardless of the adjuvant used (IFA or CFA), 100% of the monkeys immunized with rhMOG were tested positive for serum antibodies (**Figure 6E**) against conformationally intact human MOG at levels deemed pathogenic (titer >160). In contrast, no detectable antibody binding to conformationally intact human MOG could be observed in monkeys immunized with MOG34-56 in both CFA and IFA. Marmosets immunized with myelin from WT mice emulsified in CFA had levels of anti-MOG antibodies ( $\bar{x} = 265$ ) deemed pathogenic, albeit much lower than rhMOG-sensitized monkeys ( $\bar{x} = 1472$ ). Plasma from MOG<sup>-/-</sup> myelin/CFA-immunized monkeys tested negative, except for the one animal that developed clinical symptoms of EAE and pathology. Furthermore, also naïve marmosets tested negative for anti-MOG antibodies. Sera from human myelin/CFA monkeys were not available for testing.

In conclusion, we could confirm that monkeys immunized with MOG34-56 do not produce antibodies that bind to conformationally intact MOG, but future work is needed to entirely exclude a role of antibodies in demyelination in these models.

## Discussion

The ability of animal models to accurately replicate key pathological features of MS and the understanding of the limitations to which they reflect aspects of disease, is vital in the selection process for preclinical testing of novel therapeutics<sup>9</sup>. GM injury is increasingly recognized as a key contributor to disease progression. Despite this, GM lesions are only routinely elicited in EAE in a limited number of congenic Lewis rat strains and in common marmosets<sup>10,12,13,16,22</sup>. Recently, we



E

Animal	Immunization method	Day of sacrifice	Max EAE score	MOG Titer (1:20)	MOG Titer (1:40)	Titer Final (1:)
1	rhMOG /CFA	59	2.5	+	+	2560
2		91	3	+	+	1280
3		55	3	+	+	320
4		105	2	+	+	640
5		83	3	+	+	2560
6	rhMOG/IFA	73	2.5	+	+	≥2560
7		65	3	+	+	≥2560
8		57	3	+	+	≥2560
9		81	3	+	+	≥2560
10		33	2.5	+	+	≥2560
11		26	2.5	+	+	≥2560
12		28	2.5	+	+	≥2560
13	MOG34-56/CFA	61	3	-	-	0
14		86	2.5	-	-	0
15		116	0	-	-	0
16		89	2.5	-	-	0
17		13	0	-	-	0
18	MOG34-56/IFA	85	2.5	-	-	0
19		106	2.5	-	-	0
20		85	2.5	-	-	0
21		87	2.5	-	-	0
22		51	3	-	-	0
23		62	2.5	-	-	0
24	myelin from wt mice/CFA	46	3	+	+	320
25		46	2	+	+	640
26		123	2.5	+	+	80
27		39	3	+	-	20
28	MOG <sup>-/-</sup> myelin/CFA	25	5	+	+	40
29		152	1	-	-	0
30		165	0.5	-	-	0
31		152	0.5	-	-	0
32		152	1	-	-	0

**Figure 6. IgG, complement activation and anti-MOG antibodies.** Deposition of IgG and C9neo was analyzed by immunohistochemistry. Lesions were characterized by staining of PLP (A) and MRP14 (B). In all models (A-D = MOG34-56/IFA), deposition of IgG could be observed in both white matter (WM) and grey matter (GM), regardless of the presence of lesions (C). Immunoreactivity to C9neo could readily be observed in early active lesions of WM, leukocortical and intracortical lesions, but was not easily discernable in subpial lesions (D). E, Titers of pathogenic antibodies to conformationally intact MOG in sera of EAE and non-EAE marmosets.



demonstrated that also oxidative tissue injury, a key feature of MS pathology, is well replicated in marmoset EAE<sup>23</sup>, while this pathological aspect is absent in almost all rodent EAE models tested thus far<sup>24</sup>. The conclusion is therefore warranted that the marmoset EAE model, of all known EAE models, most comprehensively approximates the pathological complexity of MS.

In the current study, we performed a comparative analysis of different EAE induction protocols for their ability to elicit WM and GM injury in the marmoset. Whereas previously we estimated GM injury in marmosets to occur in roughly 10-20% of the tested marmosets (our own observation), the actually observed numbers far exceeded this. The discrepancy between previously estimated and actually observed prevalence of GM injury, could be due to the reliance on MRI when estimating the proportion of animals that developed GM injury. Whereas leukocortical or large inflamed intracortical lesions may be detected on routine T2W MR images, subpial lesions are frequently missed in MS<sup>25</sup>.

One aim of this study was to gain insight into the mechanisms involved in GM lesion formation. The results of this study demonstrated that the method with which EAE is induced determines the development of GM injury. When comparing the role of the adjuvant, marmosets immunized with CFA-based antigen formulations, irrespective of the antigen used, had significantly more GM injury. This was also true when the high responding human myelin/CFA-immunized monkeys were excluded from the analysis. Interestingly, the role of adjuvant was clear in marmosets immunized with rhMOG, but less outspoken in marmosets immunized with MOG34-56. This observation may be explained by the role of innate immunity in GM injury in rhMOG-induced models, where anti-MOG antibodies are involved. Recently it has been demonstrated by others that depletion of CCR2+ monocytes in rhMOG/CFA-immunized marmosets significantly reduced subpial demyelination and disease severity<sup>26</sup>. Whereas the additional innate stimuli given by CFA could enhance antibody dependent cell-mediated cytotoxicity in models where a clear role of antibodies has been established; it is tempting to speculate that the role of adjuvant is less pertinent in MOG34-56 immunized marmosets, because these models are based on the reactivation of effector memory cytotoxic T lymphocytes.

One caveat to this analysis is the considerable heterogeneity with respect to the amount of GM demyelination, even between individual monkeys in the same protocol. This finding is consistent with what is observed in the MS patient<sup>27</sup>. This suggests that other factors besides the EAE induction method may strongly influence GM pathology development, such as tissue vulnerability. In the MS patient, GM pathology becomes more evident as disease progresses and brain atrophy is associated with later stages of disease; hence duration and severity of the disease may contribute to the degree to which the GM is injured in the marmoset model<sup>28</sup>. Indeed, the model that exhibited the most severe GM injury was the human myelin/CFA group which had a longer duration/time between immunization and the development of clinical symptoms suggesting chronicity of disease plays a role in GM lesion formation<sup>21</sup>.

Other potential rate-limiting factors in GM injury could be MHC (Caja) allelic variation, and lymphocryptovirus infection status. As the development of GM lesions in Lewis rat is dictated by the MHC variation, and it has been postulated that the CalHV3 infected B cell is the obligatory antigen-presenting cell in marmoset EAE, future investigation is warranted<sup>29</sup>.



One interesting observation of this study was the finding that the correlation between WM and GM injury in marmoset EAE was dependent upon the model used. There was a correlation in marmosets immunized with rhMOG, but no positive correlation was observed in other protocols. Focal demyelination of the WM does not correlate to injury of the GM in the MS patient<sup>25,30</sup>. However, a weak correlation between the amount of WM inflammation and GM demyelination has been demonstrated<sup>28</sup>. The current study did not address the correlation between WM inflammation and GM demyelination, but it should be noted that we have observed both extremes of the spectrum; i.e. monkeys with massive GM injury and widespread WM inflammation with minimal WM demyelination, and those with widespread WM demyelination and inflammation, but negligible GM injury. One should interpret this data with slight caution, however, as WM demyelination was quantified on the basis of PLP staining. Whereas MOG has shown to be sequentially lost earlier than PLP, detection of cortical lesions on the basis of MOG has technical difficulties<sup>31</sup>. Thus, the true amount of demyelination would likely be much higher in the WM of marmosets that contained many early active lesions when assessing demyelination based on MOG.

Finally, we confirmed previous reports documenting that in MOG34-56 immunized marmosets pathogenic antibodies against conformationally intact MOG are not formed<sup>14,32</sup>. A role of antibodies of other specificities than MOG in demyelination has yet to be excluded. As C9neo deposition was observed only in early active lesions, it is unclear if this staining pattern represents a technical artifact as detailed by Prineas and colleagues<sup>33</sup> or if a non-MOG pathogenic antibody plays a role in disease progression. A future investigation to provide clarity on the detection of this staining pattern in the early active lesion is warranted.

In conclusion, we demonstrate that demyelination of the GM is highly variable in the marmoset EAE model. GM injury in NHP can be moderately influenced by the method of EAE induction, and is also likely highly influenced by factors not assessed in this analysis. Like in humans, the formation of GM lesions in marmosets is at least partially dissociated from WM demyelination, particularly in the peptide-based models. Together, these results suggest that in at least a cohort of marmosets, a more progressive MS-like pathology occurs.

## Acknowledgement

The authors like to thank F. van Hassel for the artwork. J. Dunham was funded by the European Union with a Marie Curie Fellowship (NeuroKine; 316722), and the authors are grateful for this support.



## References

- 1 Flachenecker, P. & Stuke, K. National MS registries. *J Neurol* **255 Suppl 6**, 102-108, doi:10.1007/s00415-008-6019-5 (2008).
- 2 Popescu, B. F., Pirko, I. & Lucchinetti, C. F. Pathology of multiple sclerosis: where do we stand? *Continuum (Minneapolis)* **19**, 901-921, doi:10.1212/01.CON.0000433291.23091.65 (2013).
- 3 Geurts, J. J. & Barkhof, F. Grey matter pathology in multiple sclerosis. *Lancet Neurol* **7**, 841-851, doi:10.1016/S1474-4422(08)70191-1 (2008).
- 4 Amato, M. P. *et al.* Association of neocortical volume changes with cognitive deterioration in relapsing-remitting multiple sclerosis. *Arch Neurol* **64**, 1157-1161, doi:10.1001/archneur.64.8.1157 (2007).
- 5 Amato, M. P. *et al.* Neocortical volume decrease in relapsing-remitting MS patients with mild cognitive impairment. *Neurology* **63**, 89-93 (2004).
- 6 Charil, A. *et al.* Focal cortical atrophy in multiple sclerosis: relation to lesion load and disability. *Neuroimage* **34**, 509-517, doi:10.1016/j.neuroimage.2006.10.006 (2007).
- 7 Bo, L., Vedeler, C. A., Nyland, H. I., Trapp, B. D. & Mork, S. J. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. *J Neuropathol Exp Neurol* **62**, 723-732 (2003).
- 8 Haider, L. *et al.* The topography of demyelination and neurodegeneration in the multiple sclerosis brain. *Brain* **139**, 807-815, doi:10.1093/brain/awv398 (2016).
- 9 Ransohoff, R. M. Animal models of multiple sclerosis: the good, the bad and the bottom line. *Nat Neurosci* **15**, 1074-1077, doi:10.1038/nn.3168 (2012).
- 10 Merkler, D., Ernsting, T., Kerschensteiner, M., Brück, W. & Stadelmann, C. A new focal EAE model of cortical demyelination: multiple sclerosis-like lesions with rapid resolution of inflammation and extensive remyelination. *Brain* **129**, 1972-1983, doi:10.1093/brain/awl135 (2006).
- 11 Huizinga, R., Gerritsen, W., Heijmans, N. & Amor, S. Axonal loss and gray matter pathology as a direct result of autoimmunity to neurofilaments. *Neurobiol Dis* **32**, 461-470, doi:10.1016/j.nbd.2008.08.009 (2008).
- 12 Storch, M. K. *et al.* Cortical demyelination can be modeled in specific rat models of autoimmune encephalomyelitis and is major histocompatibility complex (MHC) haplotype-related. *J Neuropathol Exp Neurol* **65**, 1137-1142, doi:10.1097/01.jnen.0000248547.13176.9d (2006).
- 13 Gardner, C. *et al.* Cortical grey matter demyelination can be induced by elevated pro-inflammatory cytokines in the subarachnoid space of MOG-immunized rats. *Brain* **136**, 3596-3608, doi:10.1093/brain/awt279 (2013).
- 14 Jagessar, S. A. *et al.* Induction of progressive demyelinating autoimmune encephalomyelitis in common marmoset monkeys using MOG34-56 peptide in incomplete Freund adjuvant. *J Neuropathol Exp Neurol* **69**, 372-385, doi:10.1097/NEN.0b013e3181d5d053 (2010).
- 15 Haanstra, K. G. *et al.* Induction of experimental autoimmune encephalomyelitis with recombinant human myelin oligodendrocyte glycoprotein in incomplete Freund's adjuvant in three non-human primate species. *J Neuroimmune Pharmacol* **8**, 1251-1264, doi:10.1007/s11481-013-9487-z (2013).
- 16 Pomeroy, I. M., Jordan, E. K., Frank, J. A., Matthews, P. M. & Esiri, M. M. Diffuse cortical atrophy in a marmoset model of multiple sclerosis. *Neurosci Lett* **437**, 121-124, doi:10.1016/j.neulet.2008.03.069 (2008).
- 17 Pomeroy, I. M., Matthews, P. M., Frank, J. A., Jordan, E. K. & Esiri, M. M. Demyelinated neocortical lesions in marmoset autoimmune encephalomyelitis mimic those in multiple sclerosis. *Brain* **128**, 2713-2721, doi:10.1093/brain/awh626 (2005).
- 18 Jagessar, S. A. *et al.* Overview of models, methods, and reagents developed for translational autoimmunity research in the common marmoset (*Callithrix jacchus*). *Exp Anim* **62**, 159-171 (2013).
- 19 Mader, S. *et al.* Complement activating antibodies to myelin oligodendrocyte glycoprotein in neuromyelitis optica and related disorders. *J Neuroinflammation* **8**, 184, doi:10.1186/1742-2094-8-184 (2011).
- 20 Di Pauli, F. *et al.* Temporal dynamics of anti-MOG antibodies in CNS demyelinating diseases. *Clin Immunol* **138**, 247-254, doi:10.1016/j.clim.2010.11.013 (2011).
- 21 't Hart, B. A. *et al.* Histopathological characterization of magnetic resonance imaging-detectable brain white matter lesions in a primate model of multiple sclerosis: a correlative study in the experimental autoimmune encephalomyelitis model in common marmosets (*Callithrix jacchus*). *Am J Pathol* **153**, 649-663 (1998).

- 22 Kap, Y. S., Jagessar, S. A., Dunham, J. & 't Hart, B. A. The common marmoset as an indispensable animal model for immunotherapy development in multiple sclerosis. *Drug Discov Today* **21**, 1200-1205, doi:10.1016/j.drudis.2016.03.014 (2016).
- 23 Dunham, J. *et al.* Oxidative Injury and Iron Redistribution Are Pathological Hallmarks of Marmoset Experimental Autoimmune Encephalomyelitis. *J Neuropathol Exp Neurol*, doi:10.1093/jnen/nlx034 (2017).
- 24 Schuh, C. *et al.* Oxidative tissue injury in multiple sclerosis is only partly reflected in experimental disease models. *Acta Neuropathol* **128**, 247-266, doi:10.1007/s00401-014-1263-5 (2014).
- 25 Geurts, J. J. *et al.* Cortical lesions in multiple sclerosis: combined postmortem MR imaging and histopathology. *AJNR Am J Neuroradiol* **26**, 572-577 (2005).
- 26 Lagumersindez-Denis, N. *et al.* Differential contribution of immune effector mechanisms to cortical demyelination in multiple sclerosis. *Acta Neuropathol*, doi:10.1007/s00401-017-1706-x (2017).
- 27 Vercellino, M. *et al.* Grey matter pathology in multiple sclerosis. *J Neuropathol Exp Neurol* **64**, 1101-1107 (2005).
- 28 Kutzelnigg, A. *et al.* Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain* **128**, 2705-2712, doi:10.1093/brain/awh641 (2005).
- 29 't Hart, B. A., Kap, Y. S., Morandi, E., Laman, J. D. & Gran, B. EBV Infection and Multiple Sclerosis: Lessons from a Marmoset Model. *Trends Mol Med* **22**, 1012-1024, doi:10.1016/j.molmed.2016.10.007 (2016).
- 30 Bo, L., Geurts, J. J., van der Valk, P., Polman, C. & Barkhof, F. Lack of correlation between cortical demyelination and white matter pathologic changes in multiple sclerosis. *Arch Neurol* **64**, 76-80, doi:10.1001/archneur.64.1.76 (2007).
- 31 van der Valk, P. & De Groot, C. J. Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. *Neuropathol Appl Neurobiol* **26**, 2-10 (2000).
- 32 Kap, Y. S. *et al.* Fast progression of recombinant human myelin/oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in marmosets is associated with the activation of MOG34-56-specific cytotoxic T cells. *J Immunol* **180**, 1326-1337 (2008).
- 33 Barnett, M. H., Parratt, J. D., Cho, E. S. & Prineas, J. W. Immunoglobulins and complement in postmortem multiple sclerosis tissue. *Ann Neurol* **65**, 32-46, doi:10.1002/ana.21524 (2009).





