3.1

Oxidative injury and iron redistribution are pathological hallmarks of marmoset experimental autoimmune encephalomyelitis

Jordon Dunham1,2, Jan Bauer3, Graham R. Campbell4, Don J. Mahad4, Nikki van Driel1, Susanne M.A. van der Pol5, Bert A. ‘t Hart1,2, Hans Lassmann3, Jon D. Laman2, Jack van Horssen5,* and Yolanda S. Kap1,*

1Dept. of Immunobiology, Biomedical Primate Research Centre, Rijswijk, The Netherlands; 2University of Groningen, University Medical Center, Dept. of Neuroscience, Groningen, The Netherlands; 3Medical University of Vienna, Center for Brain Research, Vienna, Austria; 4Centre for Neuroregeneration, University of Edinburgh, Edinburgh, United Kingdom. 5Dept. of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands

*Shared senior authorship
Abstract

Oxidative damage and iron redistribution are associated with multiple sclerosis (MS) disease pathogenesis and progression, but these aspects are not entirely replicated in rodent experimental autoimmune encephalomyelitis (EAE) models. Here, we report that oxidative burst and injury as well as redistribution of iron are hallmarks of the MS-like pathology in the EAE model in the common marmoset. Active lesions in the marmoset EAE brain display increased expression of NADPH oxidase (p22phox, p47phox, gp91phox) and inducible nitric oxide synthase immunoreactivity within lesions with active inflammation and demyelination, coinciding with enhanced expression of mitochondrial heat shock protein 70 and superoxide dismutase 1 and 2. The EAE lesion-associated liberation of iron, due to loss of iron containing myelin, was associated with altered expression of iron metabolic markers (FtH1, lactoferrin, hephaestin, ceruloplasmin). The enhanced expression of oxidative damage markers in inflammatory lesions indicates that the enhanced antioxidant enzyme expression could not counteract reactive oxygen and nitrogen species-induced cellular damage similarly as observed in MS brains. In conclusion, this study demonstrates that oxidative injury and aberrant iron distribution are prominent pathological hallmarks of marmoset EAE thus making this model suitable for therapeutic intervention studies aimed at reducing oxidative stress and associated iron dysmetabolism.
Introduction

The development of adequate treatments for progressive multiple sclerosis (MS) is hampered by the lack of an animal model that accurately reproduces pathological hallmarks of the human disease. Recent attention has focused on oxidative damage, mitochondrial dysfunction, and energy deficits as main causes of tissue damage and diminished re-myelination capacity during progression of multiple sclerosis (MS) (reviewed in 1,2). However, these features are insufficiently represented in rodent models for MS, i.e. experimental autoimmune encephalomyelitis (EAE) 3-5.

Tissue damage by reactive oxygen species (ROS) is an important cytotoxic effector mechanism of myeloid immune cells, such as macrophages. The cascade-wise production of ROS starts with the activation of NADPH oxidase-2 (NOX2), which in active MS lesions is highly expressed by microglia and macrophages. The activation of NOX2 involves the translocation and assembly of cytosolic (p47phox and p67phox) with membrane-bound (gp91phox, p22phox) subunits into a multimeric complex expressed in membranes of phagolysosomes and the cell surface 6-8. The function of NOX2 is transfer of electrons from NADPH to molecular oxygen, yielding superoxide anion (O2\(^{-}\)). The concomitant MS lesion-restricted expression of inducible nitric oxide synthase (iNOS) by astrocytes and macrophages produces nitric oxide that by reaction with superoxide gives the highly toxic peroxynitrite 4,9,10.

The tissue response to increased ROS involves upregulation of various scavenger molecules (e.g. superoxide dismutases; SOD) and chaperones (heat shock proteins) to limit injury. Superoxide anion is neutralized by SOD that converts the radical into the less toxic hydrogen peroxide, which is neutralized by the anti-oxidant enzyme catalase and via the glutathione peroxidase pathway. Cytosolic SOD1 and mitochondrial SOD2 are ubiquitously expressed in the central nervous system (CNS) and substantially increased in hypertrophic astrocytes and myelin-laden macrophages of the MS brain 6,9,11. Mitochondrial heat-shock protein 70 (mtHSP70) is a chaperonin induced upon a variety of stressors, including oxidative stress 12. ROS metabolites cause metabolic stress, peroxidation of lipids and proteins, and DNA alkylation, which are observed in MS lesions 13. These features, together with mitochondrial defects and corresponding energy deficits, have been implicated as crucial pathological components of disease progression 14,15.

While some aspects of oxidative stress have been observed in rodent EAE models, the characteristic lesion-restricted redistribution of iron in MS is lacking 14. The accumulation of iron in myelin and oligodendrocytes in the aging human brain can be detrimental when it is liberated upon demyelination as iron amplifies oxidative damage 16. Iron liberation in MS is associated with lesion-associated changes in iron metabolic markers, such as ferritin heavy chain (FTH1), lactoferrin, hephaestin, and ceruloplasmin 17,18.

In recent years, the EAE model in the common marmoset (Callithrix jacchus) has emerged as a translationally relevant MS model, which could bridge the gap between clinical and histopathological characteristics of murine EAE models and MS 19. CNS pathology in the marmoset EAE model typically involves both WM and grey matter (GM) demyelination. Essentially all stages of WM lesion development ranging from early active lesions with myelin-laden macrophages to chronic gliotic lesions and the various types of GM lesions in MS have also been observed in marmoset EAE brain tissue 20-23.
In the current study, we have examined whether the marmoset EAE model may represent an appropriate model to investigate processes involved in oxidative injury, such as oxidative burst and changes in iron metabolism. The extensive examination of marmoset EAE brain samples reported here demonstrates widespread oxidative tissue injury and aberrant iron distribution in the marmoset EAE brain, similarly as observed in human MS brain lesions.

**Material and Methods**

**Marmoset tissue**

Cryopreserved and formalin-fixed, paraffin-embedded tissues from previous marmoset EAE experiments performed at the Biomedical Primate Research Centre were used for this study (BPRC, Rijswijk, The Netherlands). These studies were reviewed and approved by the institutional ethics review committee. For a detailed description of EAE induction and monitoring of EAE course see Jagessar et al. Briefly, marmosets were immunized once (in the case of myelin or rhMOG) or every 28 days (inoculations with MOG34-56) and monitored daily until the development of overt neurological disease (EAE score ≥ 2). An accumulative scoring system was implemented whereby a score 2 = ataxia or optic disease, score 2.5 = paresis of one or more limbs, score 3 = paralysis of one or more limbs. Ethical endpoint were in compliance with instructions of the ethics committee and are indicated for individual animals in Table 1. Brain tissues were derived from EAE models induced with myelin emulsified in CFA, human recombinant myelin oligodendrocyte glycoprotein (rhMOG) emulsified in CFA (rhMOG/CFA), and MOG peptide 34-56 emulsified in either CFA (MOG34-56/CFA) or IFA (MOG34-56/IFA). Half brains were sectioned (coronal) at multiple locations during the embedding process; hence different topographic regions of tissue could be analyzed at once on one slide. Marmosets (n=24) selected for this study were previously characterized for brain pathology, and featured prominent WM and GM pathology. The predilection sites of lesions in marmoset EAE models include, but are not limited to, corpus callosum and areas close to the ventricles, yet demyelination is widespread. Representative lesions from coronal sections from mid-brain areas are shown.

**Immunohistochemistry**

For immunohistochemical stainings of formalin-fixed paraffin embedded material, 5 µm sections were cut and deparaffinized using xylene (VWR, Radnor, PA) and rehydrated via graded ethanol. Endogenous peroxidase activity was blocked by incubating tissue in 0.03% H₂O₂ (Sigma, St. Louis, MO) in methanol for 30 min. A pretreatment step was performed for antigen retrieval by steaming slides in either EDTA (pH 8.6; Sigma) or citrate (pH 6.0; Sigma). For cryo-preserved preserved material, sections of 6-8 µm were mounted onto permafrost plus tissue slides, prior to a fixation step with acetone (10 min), or 4% PFA (30 min). Non-specific antibody binding was blocked by pre-incubation with 10% FCS in Dako wash buffer (DAKO, Glostrup, Denmark).

Following blocking of non-specific Ig binding, sections were incubated with primary antibodies (See Table 2) overnight at 4°C. Biotin-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and avidin-labeled peroxidase were added prior to visualization with diaminobenzidine tetrachloride (Sigma). A hemalaun counterstain was performed by 2 min
incubation with 1:10 diluted hemalaun (Merck Millipore; Billerica, MA). Finally, tissue was dehydrated with graded ethanol and xylene prior to mounting with malinol (Waldeck, Münster, Germany).

For double or triple fluorescent labeling, the above protocol was used, with minor deviations. Briefly, following overnight incubation at 4°C with primary antibodies diluted in Dako ready-to-use diluent (DAKO) or TBS with 10% FCS, slides were washed in TBS and incubated for an additional 1 h. For visualization, direct secondary antibodies against primary host species (mouse, goat, or sheep) conjugated to CY3 and CY5 (Jackson ImmunoResearch Laboratories,) were used. For primary antibodies with rabbit host, a secondary biotin anti-rabbit antibody (Jackson ImmunoResearch Laboratories) was used prior to labeling with a Cy2 conjugated streptavidin (Jackson ImmunoResearch Laboratories). Cell nuclei were visualized using a commercially available Vectashield DAPI antifade mounting kit (Vector laboratories, Burlingame, CA). Fluorescence detection was performed with a Leica systems microscope (DMI 600B; Leica Microsystems GmbH, Wetzlar, Germany) using a 63x immersion oil lense.

Table 1. Animals used in this study

<table>
<thead>
<tr>
<th>Animal</th>
<th>Gender</th>
<th>Age (months)a</th>
<th>Immunization</th>
<th>Week of sacrifice</th>
<th>Max. EAE score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>57</td>
<td>Myelin/CFA</td>
<td>76</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>33</td>
<td>Myelin/CFA</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>45</td>
<td>Myelin/CFA</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>27</td>
<td>rhMOG/CFA</td>
<td>21</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>28</td>
<td>rhMOG/CFA</td>
<td>16</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>28</td>
<td>rhMOG/CFA</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>43</td>
<td>rhMOG/CFA</td>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>38</td>
<td>rhMOG/CFA</td>
<td>11</td>
<td>2.5</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>21</td>
<td>MOG34-56/CFA</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>23</td>
<td>MOG34-56/CFA</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>25</td>
<td>MOG34-56/CFA</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>39</td>
<td>MOG34-56/CFA</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>39</td>
<td>MOG34-56/CFA</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>28</td>
<td>MOG34-56/CFA</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>31</td>
<td>MOG34-56/IFA</td>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>23</td>
<td>MOG34-56/IFA</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>24</td>
<td>MOG34-56/IFA</td>
<td>14</td>
<td>2.5</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>59</td>
<td>MOG34-56/IFA</td>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>50</td>
<td>MOG34-56/IFA</td>
<td>17</td>
<td>2.5</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>36</td>
<td>MOG34-56/IFA</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>30</td>
<td>MOG34-56/IFA</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>39</td>
<td>MOG34-56/IFA</td>
<td>8</td>
<td>2.5</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>68</td>
<td>MOG34-56/IFA</td>
<td>12</td>
<td>2.5</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>50</td>
<td>rhMOG/IFA</td>
<td>3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a Age at the end of the study
b Animals 9-20 were used for quantification of iron load, p22phox and iNOS
To compare lesion content of p22phox and iNOS expression, images of active lesions (n=3 lesions/marmoset) were converted to 8 bit using ImageJ. A threshold was applied to images to eliminate non-specific staining, and the binary watershed feature was utilized to distinguish individual cells as clustering was often observed. Standard scale bars were used to calculate the area of the images and data is presented as the average (n=3) of positive cells/mm² in an active WM lesion.

Iron staining

DAB-enhanced Turnbull staining of non-heme tissue (n=12 marmosets, Table 1) iron was performed on formalin-fixed paraffin-embedded tissue sections as described previously 29,30. Briefly, 5 μm tissue sections were deparaffinized using xylene, rehydrated via graded ethanol, and treated with 10% ammonium sulfide (Merk Millipore; NY) for 1.5 h and potassium-ferricyanide (Sigma) for 15 min. Endogenous peroxidase was blocked with 0.3% H$_2$O$_2$ (Sigma) prior to amplification with 0.025% 3,3'-diaminobenzidine (Sigma). Hemalaun (Merk) counterstaining was performed as described above.

For quantification purposes samples (n=12 marmosets) were stained simultaneously. Quantification was performed as described previously 17 on tissue from the MOG34-56 CFA (n=6) & IFA (n=6) groups; representing the most recent monkeys used in studies. Briefly, images (400x) of NAWM (n=2/marmoset) were color calibrated/deconvoluted to separate hematoxylin staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog</th>
<th>Host</th>
<th>Dilution</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td>DAKO</td>
<td>M0775</td>
<td>Mouse</td>
<td>1:200</td>
<td>Macrophage/microglia</td>
</tr>
<tr>
<td>TPP</td>
<td>Abcam</td>
<td>ab105033</td>
<td>Goat</td>
<td>1:100</td>
<td>Oligodendrocyte</td>
</tr>
<tr>
<td>MRP14</td>
<td>BMA biomedicals</td>
<td>S100A9</td>
<td>Mouse</td>
<td>1:250</td>
<td>Macrophage</td>
</tr>
<tr>
<td>PLP</td>
<td>Serotec</td>
<td>MCA839G</td>
<td>Mouse</td>
<td>1:200</td>
<td>Myelin</td>
</tr>
<tr>
<td>GFAP</td>
<td>Sigma</td>
<td>SAB5201104</td>
<td>Mouse</td>
<td>1:200</td>
<td>Astrocyte</td>
</tr>
<tr>
<td>NeuN</td>
<td>Millipore</td>
<td>MAB377</td>
<td>Mouse</td>
<td>1:400</td>
<td>Neuron</td>
</tr>
<tr>
<td>P22phox</td>
<td>Santa-cruz</td>
<td>SC20781</td>
<td>Rabbit</td>
<td>1:100</td>
<td>NADPH oxidase subunit</td>
</tr>
<tr>
<td>p47phox</td>
<td>Abcam</td>
<td>ab74095</td>
<td>Goat</td>
<td>1:100</td>
<td>NADPH oxidase subunit</td>
</tr>
<tr>
<td>gp91phox</td>
<td>Verhoeven et al., 1989</td>
<td>-</td>
<td>Mouse</td>
<td>1:100</td>
<td>NADPH oxidase subunit</td>
</tr>
<tr>
<td>iNOS</td>
<td>Chemicon</td>
<td>AB16311</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>mtHSP70</td>
<td>ThermoFisher</td>
<td>MA3-028</td>
<td>Mouse</td>
<td>1:100</td>
<td>Oxidative stress (mitochondria)</td>
</tr>
<tr>
<td>SOD1</td>
<td>Abcam</td>
<td>ab62800</td>
<td>Goat</td>
<td>1:250</td>
<td>Oxidative stress (cytoplasmic)</td>
</tr>
<tr>
<td>SOD2</td>
<td>Abcam</td>
<td>ab13533</td>
<td>Rabbit</td>
<td>1:250</td>
<td>Oxidative Stress (mitochondria)</td>
</tr>
<tr>
<td>Hephaestin</td>
<td>Thermofisher</td>
<td>PA5-34844</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Iron (ferroxidase)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Abcam</td>
<td>ab110449</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Iron (ferroxidase)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Thermofisher</td>
<td>PA5-29744</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Iron (iron transport and storage)</td>
</tr>
<tr>
<td>FTH1</td>
<td>Abcam</td>
<td>ab75972</td>
<td>Rabbit</td>
<td>1:250</td>
<td>Iron (iron binding)</td>
</tr>
<tr>
<td>MDA-2</td>
<td>Abcam</td>
<td>AB6463</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>3-Nitrotyrosine</td>
<td>Abcam</td>
<td>AB61392</td>
<td>Mouse</td>
<td>1:100</td>
<td>Oxidative Injury</td>
</tr>
<tr>
<td>4-HNE</td>
<td>Abcam</td>
<td>ab66545</td>
<td>Rabbit</td>
<td>1:250</td>
<td>Oxidative Injury</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>Abcam</td>
<td>ab62623</td>
<td>Mouse</td>
<td>1:200</td>
<td>Oxidative Injury</td>
</tr>
</tbody>
</table>
from DAB staining. The mean grey value (sum of grey values of all pixels/number of pixels) was recorded for each image and used to calculate optical density (log(max intensity/mean grey value)).

**Artwork and statistics**

Figures were compiled using Adobe InDesign CC 2015 (Adobe Systems, San Jose, CA) and in some cases images were adjusted for brightness using Adobe Photoshop CC 2015 (Adobe Systems). Statistics (Mann-Whitney or linear regression correlation) was performed using Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

**Results**

**NADPH and iNOS oxidase expression in white matter lesions**

We observed that iNOS was strongly expressed in astrocytes and MRP14-positive microglia and in recently infiltrated macrophages in active WM lesions (WML); iNOS expression was absent in NAWM (Figure 1A-D, K-L). Active lesions were characterized by extensive macrophage/microglial expression of p22phox and gp91phox, whereas both NADPH oxidase subunits were only weakly expressed in microglia in normal appearing white matter (NAWM) (Figure 1E-H, M). In active WM lesions, p47phox was profoundly expressed in gp91phox-positive microglia/macrophages and virtually absent in the NAWM (Figure 1I-J, N).

Previous work showed that marmoset EAE can not only be induced with CFA as adjuvant, but also by using IFA, an adjuvant lacking microbial components that relay danger signals to myeloid antigen presenting cells. Hence, we quantified the number of cells expressing iNOS and p22phox in active WM lesions of animals immunized with MOG34-56 in either CFA or IFA. No differences in the number of cells expressing iNOS and p22phox were observed between the CFA and IFA groups (Figure 1O-P).

**iNOS and NADPH oxidase expression in grey matter lesions**

Expression of iNOS and p22phox was virtually absent in NAGM (Figure 2A-D), but strikingly upregulated in active intracortical and leukocortical lesions. iNOS and p22phox expression was generally less abundant in intracortical lesions compared to WM lesions and leukocortical lesions (Figure 2G-J, M-P). Overall, only a limited number of iNOS- and p22phox-immunoreactive cells were observed in inactive or late active subpial lesions (Figure 2S-V). In conclusion, our data show that iNOS and p22phox were highly upregulated in inflamed GM lesions in the marmoset EAE brain.

**Enhanced expression of anti-oxidant enzymes indicate oxidative stress**

We investigated whether the enhanced expression of enzymes involved in free radical production in marmoset EAE lesions might coincide with induction of endogenous antioxidants, such as SOD1 and SOD2. Both cytosolic SOD1 and mitochondrial SOD2 were weakly expressed in glial cells in NAWM, but markedly upregulated in active lesions (Figure 3A-F). SOD2 expression was observed in astrocytes, macrophages/microglia, axons and neurons (Figure 3I-K). Parenchymal SOD2 expression was markedly higher, at a basal level, in the NAGM (Figure 2E).
Figure 1. NADPH oxidase and iNOS are expressed in WM lesions. Expression of NADPH and nitric oxide synthase were observed in areas of active demyelination. Active lesions were demonstrated by a loss of myelin (PLP, A), macrophage uptake of PLP (inset of a), and the presence of infiltrated macrophages (MRP14, B). The dotted yellow line indicates the border between the WM lesion and the NAWM. No iNOS was observed in NAWM (C), but strongly expressed in active lesions (D). Expression of membrane subunits of the NADPH oxidase complex, p22phox (E-F) and gp91phox (G-H), was sparse in the NAWM, but high in active lesions. Cytosolic member, p47phox, indicative of a functional NADPH oxidase, exhibited a sparse expression in NAWM (I) and strong expression in the active lesion (J). Fluorescent labeling of iNOS (green, K-L, nuclei are grey), revealed strong expression in astrocytes (K, red), and macrophages (L, red), but, as expected, not in oligodendrocytes (K, blue). Expression of p22phox (green, M, nuclei are grey) was not observed in astrocytes or oligodendrocytes (data not shown), but strongly detectable in macrophages (M, MRP14= red). Co-localization of p47phox (green) and membrane subunit gp91phox (red) was observed (N, nuclei=blue). Similar expression of iNOS (O) and p22phox (P) was observed in MOG34-56/IFA-immunized marmosets compared to MOG34-56/CFA-immunized marmosets, implying that mycobacterial components in the immunization inoculum are not (solely) responsible for the activation of the ROS/RNS pathway. Image scale bars are 200 μm (open square), 100 μm (closed square) and 50 μm (closed circle).
Figure 2. Oxidative stress markers in cortical lesions. Oxidative stress was examined in NAGM (A-F), leukocortical (G-L), intracortical (M-R) and subpial lesions (S-X). Activity of the lesion was determined by the expression of PLP (A, G, M, S) and MRP14 or HLA-DR (B, H, N, T). Expression of p22phox (I, O) and iNOS (J, P) was observed in active leukocortical and intracortical lesions. Demyelinated subpial regions were either chronic active or inactive and devoid of p22phox (U) and iNOS (V) expression. Oxidative stress markers SOD2 (E) and mtHSP70 (F) were detected at higher basal levels in the GM compared to WM areas, with increased expression in leukocortical (K, Q) and intracortical lesions (L, R). No noticeable changes in expression of SOD2 (W) or mtHSP70 (X) were observed in the subpial lesion. Image scale bars are 200 μm (open square) and 50 μm (closed circle).
Figure 3. Oxidative stress markers are expressed in the marmoset EAE brain. Immunohistochemical detection of classical markers of oxidative stress was performed on marmoset EAE brain tissue. Depicted are PLP (A) and inflammation (MRP14; B) showing active demyelination with uptake of PLP by macrophages (insert of A). The dotted yellow line indicates the border between the WM lesion and the NAWM. Expression of SOD1 was weak or absent in NAWM (C) and upregulated in the lesion (D). Expression of SOD2 (C-F) and mtHSP70 (G-H) was clearly observed in NAWM (E, G) and markedly upregulated within the active lesion (F, H) and in areas proximal to inflammation. The punctate staining pattern of mtHSP70 and SOD2 suggests mitochondrial immunoreactivity. Expression of SOD2 (green) was observed in astrocytes (red, I), macrophages (red, J), neurons (red, K) and axons (red, inset K), but much less frequently in oligodendrocytes (blue, J). Expression of mtHSP70 (green) was sparse in astrocytes (red, L). Strong expression of mtHSP70 was observed in macrophages (red, M) and oligodendrocytes (dark blue, L-M). Co-localization of SOD2 (green) and mtHSP70 (red) was frequently observed (N). Image scale bars are 200 μm (open square), 100 μm (closed square) and 50 μm (closed circle).
Oxidative damage in marmoset EAE

than NAWM (Figure 3E). Compared to NAGM and inactive subpial lesions, SOD2 expression was strongly upregulated in active leukocortical and intracortical lesions (Figure 2K, Q, W).

Heat-shock protein expression confirms stressed mitochondria

The expression pattern of mtHSP70 closely mirrored that of SOD2 with a clear punctate staining pattern indicative of mitochondrial localization (Figure 3G-H). mtHSP70 immunoreactivity was markedly upregulated in active lesions compared to NAWM (Figure 3G-H). The expression of mtHSP70 was predominantly observed in oligodendrocytes and macrophages/microglia, but relatively less frequent in astrocytes (Figure 3L-M). Similar to SOD2, strong expression was observed in cells of neuronal morphology and in axons. Co-localization of SOD2 and mtHSP70 was frequently observed (Figure 3N) in macrophages/microglia or neurons. With respect to cortical regions of the brain, similar to SOD2, the NAGM exhibited a higher level of basal expression of mtHPS70 than the NAWM (Figure 2F). In the active leukocortical and intracortical lesion, mtHSP70 was upregulated (Figure 2L, R). Likewise, subpial lesion exhibited no changes with respect to mtHSP70 expression compared to similar areas of NAGM (Figure 2X). Together, these data demonstrate that the inflamed marmoset EAE brain is under severe oxidative stress and that endogenous anti-oxidant factors (SOD1-2) and mitochondrial heat shock proteins are markedly upregulated in active WM and cortical GM lesions.

Iron in the marmoset brain

Since iron can catalyze oxidative stress, we evaluated the presence of total non-heme iron in the marmoset brain by analyzing Turnbull stainings of paraffin-embedded tissue sections. The marmoset EAE brain exhibited considerable accumulation of total non-heme iron in myelin and oligodendrocytes in NAWM. In demyelinated WM lesions, redistribution of iron was observed, probably caused by the loss of myelin (Figure 4C-F). A general pattern of iron loss increasing towards the center of the lesion was observed. This correlated to myelin loss and was accompanied by iron uptake in macrophages/microglia and residual oligodendrocytes (Figure 4C-F).

As iron accumulates in the human brain in an ageing-associated manner, we analyzed the iron content in relation to age of the marmoset. There was a significant correlation between the age of the marmoset and iron accumulation (Figure 4G). Marmosets, mature at 15 months of age, are typically used for EAE studies upon 2 years old. The age range of EAE marmosets (n=12) in which iron was quantified was from 21-59 months, representing young sexually mature monkeys in prime adult years. When data was further analyzed by grouping marmosets based upon age as very young (≤25 months, mean= 21.6) versus middle aged monkeys (≥25 months, mean= 40.5) a clear age-associated iron accumulation was observed (Figure 4G, H).

In conclusion, our data demonstrated age-associated iron accumulation and redistribution in marmoset EAE brain tissue similarly as observed in the adult human brain.

Iron metabolic markers change with demyelination

Next, we assessed the expression of various proteins involved in iron metabolism. Lactoferrin, an iron storage marker, was particularly expressed in astrocytes in the NAWM (Figure 5A-C). Lactoferrin expression was markedly upregulated in astrocytes and microglia/macrophages in
Figure 4. Iron accumulates in the marmoset brain and is liberated upon demyelination. To determine tissue specific iron, Turnbull staining was performed on marmoset EAE brain tissue in an age range representing the lower and upper limits of age used in EAE studies. Shown is an overview image of PLP (A), MRP14 (B) and Turnbull (C) stain of a middle aged (36 mo, animal 20 table 1) marmoset with both NAWM and active demyelinating areas as determined by the presence of PLP+ macrophages (A inset). Lesions are denoted by black arrows. The images of D-F are representative areas of NAWM, lesion edge and lesion center respectively. In areas of NAWM, strong accumulation of iron associated with myelin and oligodendrocytes was observed (D). Conversely, iron loads typically were highest in areas at the edge of demyelinating lesions (E) and typically lowest in lesion centers (F), corresponding to the loss of iron-containing myelin and oligodendrocytes. Iron content of the NAWM was plotted against age showing that accumulation of iron was age dependent, with older monkeys exhibiting strongest iron deposition (G-H). Image scale bars are 500 μm (perpendicular line), 50 μm (closed circle) and 50 μm (closed circle).
Figure 5. Iron metabolic markers are expressed in the marmoset EAE brain. Immunoreactivity of classic markers associated with iron metabolism such as storage (FTH1), binding (lactoferrin) and ferroxidase activity (ceruloplasmin & hephaestin) was assessed on marmoset EAE brains. NAWM was determined by the presence of intact myelin (PLP, A) and absence of MRP14 inflammation (B) in adjacent cut sections. Active demyelination was determined by the presence of PLP in macrophages (inset of A). Expression of lactoferrin was frequently observed in the NAWM (C) and markedly upregulated in the active lesion (D). Lactoferrin (green, K-L) expression in the active lesion was observed both in astrocytes (red, K) and macrophages (red, L), but not in oligodendrocytes (blue, K-L). Expression of ferritin heavy chain (FTH1) in the NAWM (E) was frequent and associated with macrophage/microglia, whereas in the active lesion (F) profound expression (FTH1=green, M-N) was observed within astrocytes (red, M) and macrophages (red, N; oligodendrocytes/TPP=blue). In the NAWM (G) hephaestin expression was sparse and, if observed, typically reserved to cells of oligodendrocyte morphology. In the active lesion (H), hephaestin (green, O-P) expression was upregulated in astrocytes (red, O) and macrophages (red, P). Ceruloplasmin was frequently expressed in the NAWM (I) and markedly upregulated in the active lesion (J) with expression (ceruloplasmin=green; Q=R) strongly being detected in macrophages (red, R), but not astrocytes (red, Q) or oligodendrocytes (dark blue, Q). Image scale bars are 500 μm (perpendicular line), 100 μm (closed square) and 50 μm (closed circle) and 20 μm (arrows). Black arrow denotes WML.
periplaque WM (PPWM) and areas of active demyelination (Figure 5D, K-L). The iron binding marker ferritin (heavy chain, FTH1) was predominantly detected in macrophages/microglia and astrocytes throughout lesional areas (Figure 5F, M-N). In contrast iron and ferritin-containing macrophages were not frequently observed in the NAWM (Figure 5E).

Hephaestin, a transmembrane copper-dependent ferroxidase (=Fe-oxido-reductase) molecule, exhibited a weak expression pattern in oligodendrocytes or occasionally in astrocytes in NAWM (Figure 5G). Active lesions showed an enhanced expression of hephaestin in in occasional residual oligodendrocytes and oligodendrocytes in PPWM (Figure 5H). In addition, hephaestin was expressed by astrocytes and macrophages (Figure 5M-N). Ceruloplasmin, a ferroxidase molecule, was present in NAWM in some microglia (Figure 5I). Ceruloplasmin immunoreactivity was markedly upregulated in PPWM and in active lesions and observed in macrophages, and occasionally in oligodendrocytes (Figure 5J, Q-R). Collectively, these data show that loss of myelin and oligodendrocytes in the marmoset EAE brain was associated with altered expression patterns of iron and iron metabolic markers and an uptake of iron in adjacent areas similarly as seen in MS brain lesions.

**Extensive oxidative damage in active marmoset EAE lesions**

The lipid peroxidation markers 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA2) were virtually absent in NAWM (Figure 6A-C, G). 4-HNE was markedly upregulated in astrocytes and macrophages/microglia in active WM lesions (Figure 6D, K-L). MDA2 was robustly expressed throughout active demyelinating lesion, particularly in macrophages (Figure 6H, M-N).

Extensive expression of 8-hydroxydeoxyguanosine (8-OHdG), a classical marker for oxidative damage to DNA, was observed in active WM lesions, but absent in NAWM (Figure 6E-F). Nitrotyrosine was weakly expressed in glial cells in the NAWM, whereas in active lesions and PPWM nitrotyrosine immunoreactivity was strongly enhanced in astrocytes and macrophages (Figure 6I-J). In conclusion, these data show extensive accumulation of oxidative damage markers in inflamed marmoset EAE lesions.

**Discussion**

Although rodent EAE models have supported the development of MS immunotherapy, they lack several clinical and pathological features of MS 4. Our current study demonstrates that the marmoset EAE model accurately replicates key aspects of MS pathology, including the occurrence of oxidative burst and associated oxidative stress and damage to essential biological macromolecules 20,22,31. MS brain lesions, particularly the active demyelinating lesions, show signs of ongoing severe oxidative stress as demonstrated by extensive accumulation of oxidized phospholipids and oxidized DNA, as well as enhanced expression of anti-oxidant factors 32. Here, we performed an in-depth histological analysis of marmoset EAE brain tissues to study the expression of key proteins and markers involved in redox and iron metabolism and we determined the extent of oxidative damage. The central message of the current study is that the expression of markers indicative of oxidative stress and injury in active demyelinating marmoset EAE lesions show a similar expression and cellular distribution pattern as active demyelinating MS lesions: 1)
Figure 6. Oxidative damage occurs in the marmoset EAE brain. Depicted are PLP (A) and inflammation (MRP14; B) showing active demyelination with uptake of PLP by macrophages (inset of A). In the NAWM (C), staining of 4-HNE was weakly expressed in some astrocytes or absent all together, but staining was markedly upregulated in active lesions (D). In the active lesion, strong 4-HNE expression (green, K-L), was observed in both astrocytes (red, K), and in macrophages (red, L). 8-OHdG was absent in NAWM (E), but could be observed in macrophages and astrocytes of the active lesion (F). MDA2 was fairly absent in NAWM (G), but markedly upregulated in the active lesion (H), with expression (green, M-E) being observed in macrophages (MRP14=red, N), but not in astrocytes (red, M). Nitrotyrosine was weakly expressed in astrocytes in NAWM (I) and markedly upregulated in active lesion immune cells and astrocytes (J). Image scale bars are 500 μm (perpendicular line), 100 μm (closed square) and 50 μm (closed circle) and 20 μm (arrows). Black arrow denotes WML.
ROS generating enzymes NADPH oxidase and iNOS are expressed in microglia/macrophages and are significantly upregulated throughout active lesions; 2) likewise, the antioxidant enzymes SOD1 and 2 decorate astrocytes and microglia/macrophages and are strongly upregulated in active lesions; 3) mitochondrial stress (mtHSP70) is markedly upregulated throughout inflamed WM and GM lesions; 4) the marmoset brain exhibits iron accumulation and displays specific alterations in iron and iron-associated metabolic markers; 5) extensive accumulation of oxidative tissue injury markers was observed throughout active demyelinating lesions.

The extent of oxidative injury is fundamentally different in rodent EAE compared to MS, with extensive oxidative injury only being observed in virus-induced models 4. Schuh and colleagues proposed several explanations for this discrepancy. NADPH oxidase and iNOS are very weakly expressed in most rodent EAE models, which might be explained by specific-pathogen-free housing that limits peripheral innate and adaptive immune stimulation. Moreover, rodent EAE models lack accumulation of iron in myelin and oligodendrocytes. Unlike rodents, marmosets are conventionally housed and exposed to environmental pathogens and thus disease induction is juxtaposed on a pathogen educated marmoset immune system 19,33. Interestingly, induction of oxidative stress did not differ per immunization protocol in marmoset EAE.

The lack of iron accumulation, together with reduced expression of enzymes involved in the production of ROS/RNS, within the rodent brain might explain the limited oxidative damage observed in rodent EAE models 4. Iron, while critical for the myelination process, is a recognized amplifier of oxidative damage 1. The importance of neurodegeneration-associated iron dysregulation in the MS brain is suggested and evidence is emerging that iron liberation is involved in lesion expansion 34. Although iron deficient mice appear to be resistant to EAE, and iron deposition has been noted in mouse EAE spinal cord tissue, overall iron deposition in the brain appears to be lacking in rodent species 5,35. Iron accumulation in older rodents, if present, appears to be limited to brain stem nuclei and is lacking in myelin tracts and oligodendrocytes 4. We here show for the first time that iron accumulates in oligodendrocytes and myelin in the marmoset brain and that EAE results in altered expression of iron metabolic markers and iron content. Iron metabolism in non-human primates (NHP), including the marmoset, closely mirrors iron metabolism of man, but differs substantially from rodents, demonstrated by the different effects of iron chelator therapies 36,37. Metabolic processing of iron in primates, and the relatively quick time to physical maturation of marmosets, in relation to most NHP species, may explain the observation that iron accumulation was observed even in the youngest monkeys examined.

In conclusion, we demonstrate that oxidative stress and injury, pathological hallmarks of the MS brain, is accurately replicated in marmoset EAE and that iron accumulation and liberation may play a role in this process. Collectively, our data imply that the marmoset is a valuable pre-clinical MS animal model, in which new therapeutics aimed at reducing oxidative damage can be explored.
Acknowledgements

The authors would like to thank F. van Hassel for the artwork. J. Dunham was funded by the European Union with a Marie Curie Fellowship (Neurokine; 316722). This project was a result from collaborative efforts between the Medical University of Vienna, University of Edinburgh, University of Groningen, VU university Medical Center and the BPRC; facilitated by the Marie Sklodowska-Curie Fellowship scheme, and the authors are grateful for this support.
References


Oxidative damage in marmoset EAE


