AGEING AND RECURRENT EPISODES OF NEUROINFLAMMATION PROMOTE PROGRESSIVE EAE IN BIOZZI ABH MICE

Short title: Ageing and inflammation contribute to progressive EAE in mice

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Abbreviations:

aEAE	acute EAE – the first neurological episode of RREAE
CNS	central nervous system
HspB5	heat shock protein B5
IBA-1	ionized calcium-binding adapter molecule 1
MC	microglial cluster
MS	multiple sclerosis
NAWM	normal-appearing white matter
NF-H	neurofilament heavy
Olig2	oligodendrocyte transcription factor 2

PEAE	progressive EAE
PLP	proteolipid protein
PPMS	primary-progressive MS
PVC	perivascular cluster
RREAE	relapsing-remitting EAE
RRMS	relapsing-remitting MS
SCH	spinal cord homogenate
SPEAE	secondary-progressive EAE
SPMS	secondary-progressive MS

Summary

Current therapies for MS reduce the frequency of relapses by modulating adaptive immune responses but fail to limit the irreversible neurodegeneration driving progressive disability. Experimental autoimmune encephalomyelitis (EAE) in Biozzi ABH mice recapitulates clinical features of MS including relapsing-remitting episodes and secondary-progressive disability. To address the contribution of recurrent inflammatory events and ageing as factors that amplify progressive neurological disease, we examined EAE in 8-12 weeks and 12 months old ABH mice. Compared to the relapsing-remitting (RREAE) and secondary progressive EAE (SPEAE) observed in young mice, old mice developed progressive disease from onset (PEAE) associated with pronounced axonal damage and increased numbers of $CD3^+$ T cells and microglia/macrophages, but not B cells. While the clinical neurological features of PEAE and SPEAE were comparable, the pathology was distinct. SPEAE was associated with significantly reduced perivascular infiltrates and T-cell numbers in the CNS as compared to PEAE and the acute phase of RREAE. In contrast to perivascular infiltrates that declined during progression from RREAE into SPEAE, the numbers of microglia clusters remained constant. Similar to what is observed during MS, the microglia clusters emerging during EAE were associated with axonal damage and oligodendrocytes expressing HspB5, but not lymphocytes. Taken together, our data reveal that the course of EAE is dependent on the age of the mice. Younger mice show a relapsing remitting phase followed by progressive disease, while old mice immediately show progression. This indicates that recurrent episodes of inflammation in the CNS, as well as age contributes to progressive neurological disease.

Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterised by inflammation associated with reversible neurological deficits and secondary progressive neurodegeneration. That these clinical phenomena may have different underlying pathologies is supported by the findings that current therapies for MS reduce the frequency of relapses, yet fail to limit the irreversible neurodegeneration that drives progressive disability (1). Similar to other neurodegenerative disorders, the onset of progressive MS is related to age, a factor known to amplify neurodegeneration (2,3). In younger people (<30 years) with MS, the disease typically begins with a relapsing-remitting phase. After 15-25 years following disease onset, or when disease is first manifested in people older than 40, MS is predominantly characterised by progressive neurological disability. Thus, the development of the progressive stage of MS may well be at least in part a consequence of ageing (4). Although the mechanisms that underlie age-associated changes in the CNS are incompletely understood, they most likely involve changes in the numbers and functions of microglia, important innate immune cells in the CNS (5). With ageing, microglia change their morphology and have a diminished capacity to migrate and phagocytose debris, known to impede regeneration and repair (5). In addition, aged microglia assume a more pro-inflammatory profile upon activation, which also augments progressive neurodegeneration (6,7). Moreover, age also influences the reparative processes such as remyelination in the CNS (8–10) as well as the ability of the adaptive immune responses to function efficiently. For example, T cells show reduced proliferation *in vitro* with age, and the functions of regulatory T cells, critical for maintaining self-tolerance, decreases at the level of activation and cytokine production (11–13).

In MS, widespread microglia/macrophage activation correlates with demyelination, axonal loss/damage and oligodendrocytes damage (14). In addition, clusters of activated HLA-DR$^+$ microglia, also termed preactive lesions (15), are present in normal-appearing white matter (NAWM) irrespective of disease duration and subtype (16). In early MS, these microglia clusters associate with axonal injury (17) while in established MS, they are associated with stressed oligodendrocytes but not with demyelination, leucocyte infiltration, axonal damage or blood-brain barrier disruption (16,18). Microglia in preactive MS lesions exhibit an intermediate phenotype characterized by the expression of markers for both the M1 and M2 state of activation (19), indicating clear differences between their activation state as compared to active lesions (20,21). Whether the association of microglial clusters with axonal damage in early MS and with oligodendrocyte stress in established MS is a function of disease duration and/or age of the patient is unknown.

The most applicable animal model to study mechanisms contributing to disease progression in MS is experimental autoimmune encephalomyelitis (EAE). While different models of EAE have been established, secondary progressive EAE (SPEAE) following a relapsing-remitting episode (RREAE) of approximately 3 months has only been reported for Biozzi ABH mice (22,23). Similar to MS, the relapsing-remitting stages of EAE are strongly associated with T- and B-cell autoimmunity (24) while the neurodegenerative component is associated with innate immunity (25).

We have previously developed an EAE model in Biozzi ABH mice in which relapsing-remitting EAE (RREAE) following immunisation of 8-12 week old mice is followed by a secondary-progressive phase (SPEAE) 3 months later (23). To better model primary progressive MS, that occurs in older people, we have induced disease in old ABH mice (12 months). In contrast to young animals, old mice immediately develop progressive

disease (PEAE) upon immunisation. We show that the increased severity of neurological disease during progressive EAE in old mice is accompanied by more severe axonal damage, increased microglia activation and enhanced infiltration by $CD3^+$ T-cells, but not by an increased appearance of microglial clusters in NAWM. Similar to what has been found during MS, microglial clusters emerging during PEAE were associated with both axonal damage and oligodendrocytes expressing HSPB5, but not with infiltrated lymphocytes.

In summary, we have developed a model of severe chronic EAE in old Biozzi ABH mice that is associated with widespread and exacerbated microglial activation in the CNS, and increased numbers of $CD3^+$ T-cells but not $B220^+$ B-cells. That the numbers of microglial clusters remain constant throughout the disease, in both old and younger mice and regardless of disease severity or extent of axonal damage, suggests that these clusters arise in response to factors other than axonal damage. Possible factors include oxidative stress or oligodendrocyte apoptosis which is associated with increased expression of HSPB5.

Materials & Methods

Induction of EAE

Biozzi ABH (H-2^{dq1}) male and female mice (originally purchased from Harlan UK ltd, Bicester UK) were bred at Queen Mary University of London under specific pathogen free conditions. Their health status was uniform throughout the studies. Mice (2 week, 3 week, 8-12 week, 8 month and 12 month old) were immunised with an emulsion of mouse spinal cord homogenate (SCH) in complete Freund's adjuvant (CFA) as described previously (23,26). The same batch of SCH was used for all experiments. Based on power analysis and sampling for pathology studies 10-22 mice per group were immunised for EAE. The studies were performed in three separate experiments to accommodate the different aged mice. As controls for each study a group of 8-12 week old mice were immunised with SCH in CFA and an aged-matched control group of mice were injected with CFA only. Control mice were sampled at corresponding time points after injection to coincide with the post-immunisation sampling days of mice with EAE.

Neurological deficits were assessed daily from day 11 onwards, and neurological signs scored on a scale from 0-5, whereby 0=normal, 1=fully flaccid tail, 2=impaired righting reflex, 3=hind-limb paresis, 4=complete hind-limb paresis and 5= moribund/death. EAE stages that were distinguished were acute (aEAE), 1^{st} remission, relapse, 2^{nd} remission, post-relapsing secondary progressive EAE (SPEAE)(23) and progressive EAE (PEAE) (Supplementary Table 1).

Animal studies were performed in compliance with UK law for care of animals and approved by the Home Office under Animals (Scientific procedures) Act 1986. We adhered to the ARRIVE guidelines for animal research in EAE (27) as described previously (23).

Immunohistochemistry

Sections (5 µm) from paraffin-embedded brains and spinal cords from different EAE stages (n=8/stage; details on the clinical history of the mice used for pathology are summarized in Supplementary Table 1.) or age-matched controls were deparaffinised in xylene, rehydrated in descending grades of alcohol and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 30 min at room temperature (RT). For antigen retrieval, sections were heated for 10 mins in Tris/ EDTA-buffer (pH 9.0) or citrate buffer (pH 6.0), after which non-specific binding was blocked for 1 h using CleanVision Blocking Solution (Immunologic, Duiven, The Netherlands) for mouse antibodies, or 5% normal goat serum (Dako, Glostrup, Denmark) in PBS when using rabbit antibodies. Sections were rinsed and incubated with primary antibodies directed to IBA-1, an ionised calcium-binding protein 1 to detect microglia/macrophages, proteolipid protein (PLP) to detect myelin integrity, the non-phosphorylated epitope of neurofilament heavy (NF-H) to detect neuronal damage, or Olig2 to detect oligodendrocytes (Table 1).

After washing, sections were incubated with appropriate horseradish peroxidase-labelled secondary antibodies (Table 1). Antibody binding was visualized using 3,3'-diaminobenzidine (Dako) and sections were rinsed in PBS. Next, the sections were treated in the microwave to remove the first antibody. Subsequently, sections were incubated with antibodies directed to IBA-1, HSPB5, CD45RA/B220 and CD3. After washing, sections were incubated with alkaline phosphatase-labelled secondary antibodies, washed in Tris-buffered saline and stained using Liquid Permanent Red (Dako). All antibodies were diluted in Antibody Diluent™ (Immunologic). Incubations were performed at room temperature in a humidified chamber. Sections were counterstained with haematoxylin and mounted with Aquatex (Millipore, Billerica, MA).

Quantitative Analysis

Microglial clusters (MC) were identified as clusters of 4 or more IBA-1^+ cells in NAWM or normal-appearing grey matter (NAGM). Perivascular clusters (PVC) were identified as clusters of 4 or more IBA-1^+ cells in close contact with blood vessels. The numbers of MC and PVC were assessed in each region, *viz.* the grey and white of the brain and spinal cord. The areas of these regions were measured using Image J (National Institutes of Health, Bethesda, MD). Images were taken with a Leica DC500 (Leica Microsystems, Heidelberg, Germany) at 1.25 x magnification. The number of pixels was counted in the regions of interest and divided by the number of pixels per 1 mm^2 to calculate the number of MC and PVC per 10 mm^2. Sections stained for IBA-1 were also double labelled for expression of the non-phosphorylated epitope of NF-H, Olig2, HSPB5, CD3 and B220. The analysis of the pathology was performed by two observers blinded to the EAE stage and disease severity.

Statistical Analysis

Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA). Comparisons of the numbers of MC and PVC present during EAE stages were performed using Kruskal-Wallis 1-way analysis of variance. The numbers per stage and region were compared pairwise using the Mann-Whitney U test. Probability values (p) of 0.05 or less were considered statistically significant.

Results

Age is a critical factor that controls the disease course of EAE

To examine the impact of age on the development of EAE, Biozzi mice of different ages were immunised with SCH in CFA. In contrast to our studies showing that 8-12-week old (young) mice develop RREAE followed by post-relapsing SPEAE about 3-5 months later (23), Biozzi ABH mice aged 2 weeks did not develop EAE (Table 2; $p<0.001$) and 3-week old mice developed disease but with a significantly reduced incidence of disease ($p < 0.01$) and disease severity ($p< 0.01$) as compared to 8-12 week old mice. Of significance was the finding that 12-month old mice consistently developed chronic disease, without a preceding relapsing remitting course as observed in the 8-12-week old mice (Table 2; clinical score 4.8 ± 0.2 *versus* 3.9 ± 0.1; $p< 0.01$). While mortality was not typically observed in young mice, a markedly increased mortality rate (8/10) was found in old mice aged 12 months.

Progressive EAE in old mice is associated with activated microglia and CD3+ T-cells

To examine the involvement of innate or adaptive responses during the different stages of EAE, spinal cord sections from mice with aEAE and CPEAE as well as age-matched controls were stained for IBA-1, CD3 and CD45RA/B220. As shown in Figure 1, IBA-1 expression in spinal cord of young control mice was significantly less prominent in both white (A, E, $p = 0.026$) and grey (B, E, $p = 0.017$) matter compared to old control mice (C,D,E). As expected, numbers of IBA-1^+ cells in mice with EAE were significantly increased in the white and grey matter as compared to control mice (for all groups $p < 0.005$; supplementary Figure 1). While no significant difference was observed in IBA-1 expression in the white matter during aEAE in young mice compared to PEAE in old mice (Figure 1F, H, J), its expression was significantly increased in the grey matter during PEAE (Figure 1G, I, J; $p = 0.0001$).

We next compared CD3 and CD45RA/B220 expression during aEAE, PEAE and SPEAE. This revealed that CD3 expression was significantly higher in white (Figure 2A, B; $p < 0.01$) and grey matter in PEAE ($p < 0.05$) compared to aEAE. To address the impact of recurrent neuroinflammatory episodes, CD3 expression in the CNS during aEAE and PEAE was compared with SPEAE. This revealed that T-cell numbers are significantly lower during SPEAE as compared to aEAE (white matter $p < 0.01$; grey matter $p < 0.01$) and PEAE (white matter $p < 0.01$; grey matter $p < 0.01$).

To examine differences between the numbers of infiltrating B cells during the different forms of EAE, the expression of the marker B220 was examined. This analysis revealed no differences in the level of B-cell infiltration of white matter regions during aEAE, PEAE and SPEAE, but did show significantly lower numbers of CD45RA/B220^+ B cells in the grey matter during SPEAE as compared to PEAE (Figure 2C; $p < 0.05$).

Microglial activation during PEAE is associated with exacerbated axonal damage in the white but not grey matter

Spinal cords from aEAE and PEAE were examined for co-expression of IBA1^+ microglia, and NF-H as a marker of axonal damage. While axonal damage was absent in control mice (Figure 3A, B), such damage was clearly observed in the subpial regions of the spinal cord white matter in young mice with aEAE (Figure 3C). In old mice with PEAE, severe axonal damage associated with numerous IBA-1^+ cells was found throughout the white matter, with only minor axonal damage in the grey matter close to the border of the white matter (**Figure 3D**).

Perivascular clusters but not microglia clusters decline with EAE progression

Given that therapies aimed at modulating adaptive immunity typically fail to limit irreversible neurodegeneration that occurs during both progressive MS (1,3) and SPEAE (24), we examined whether the extent of lymphocyte infiltration declines with disease progression, by examining the numbers of PVC and MC in NAWM and NAGM during different forms of EAE. PVC were identified as clusters of 4 or more IBA-1$^+$ cells in close contact with blood vessels (Figure 4A, B) while MC were identified as clusters of 4 or more IBA-1$^+$ cells in NAWM or NAGM and not associated with blood vessels (Figure 4C, D). PVC and MC were assessed during aEAE, RREAE and SPEAE in 8-12-week old mice and compared to PEAE in 12-month old mice. Compared to aEAE and PEAE, the numbers of PVC in the CNS in SPEAE were significantly lower (Figure 5A illustrating spinal cords, $p < 0.0029$; $p < 0.0005$; Figure 5B illustrating brains; $p < 0.0015$; $p < 0.0102$). The difference was most prominently observed in white matter regions of the spinal cord (Figure 5C) while being less marked in grey matter (Figure 5D).

In contrast to these differences in the numbers of PVC, no such differences were observed for MC (Figure 5E,F) although more microglial clusters tended to emerge in the white matter during the second remission (Figure 5G). MC were observed in 30 of 41 (73%) younger mice with EAE at all time points, and in all 12-month old mice (100%), but not in control mice. Given previous data illustrating that during MS, MC are predominantly associated with axonal damage in biopsy tissues of acute MS(17), and with stressed HSPB5$^+$ oligodendrocytes in post-mortem tissues during established MS(18), we next examined the relationship between the emergence of MC during EAE and either axonal damage or oligodendrocyte stress by staining for NF-H, olig2 and HSPB5. As shown in Figures 6A and B, IBA-1$^+$ MC were observed in NAWM in the absence as well as presence of axonal damage, and they were associated with HSPB5$^+$ olig2+ oligodendrocytes too (Figure 6C), but not with the presence of CD3$^+$ T cells or B220$^+$ B cells (Figure 6D-F).

Discussion

Current immune-modulating therapies for MS reduce the frequency of clinical relapses, yet fail to impact on the irreversible neurodegeneration driving progressive forms of MS (1) indicating an urgent unmet clinical need. The finding that age is an important risk factor for conversion from RRMS to SPMS and for the onset of PPMS indicates that senescence of the immune system and CNS are important contributors to disease progression (28). In support of a role for ageing in MS is the finding that paediatric MS is typically relapsing-remitting in nature and only rarely involves SPMS or PPMS (29).

To study pathogenic mechanisms and to support the development of potential therapeutic strategies in MS, EAE is a widely used preclinical model, but EAE models have so far provided only limited insight into the pathophysiology of progressive forms of MS. This is in part due to the frequent focus on acute EAE in C57BL/6 mice that do not develop secondary progressive disease (30). In contrast, we have previously reported that young (8-12 weeks) Biozzi ABH mice exhibit RREAE followed by SPEAE. That this model reiterates key features of SPMS including demyelination, gliosis and neuronal and axonal loss (23) indicates that this model is more suited to examine mechanisms contributing to progression of MS. To better understand the role of age and development of progressive disease we compared the EAE susceptibility of

differently aged Biozzi ABH mice. In contrast to the RREAE and SPEAE that occurs in young mice, we show that juvenile mice (2 weeks old) do not develop clinical signs of EAE while old mice (12 months) develop monophasic neurodegenerative EAE from the onset. These different clinical forms were reflected by distinct pathology in the CNS, indicating that repeated episodes of inflammation and neurological disability observed in SPEAE can be distinguished from progressive neurological disease that developed in aged individuals.

That Biozzi mice less than 2 weeks of age are fully resistant to EAE induction and 3-week old mice partially resistant may in part reflect the inadequate functional development of the immune system and pathogenic T cells including their ability to produce pro-inflammatory cytokines (31) or their ability to migrate into the CNS (32) at that young age. On the other hand, myelin-reactive T-cells from 5-week old mice have been shown to be pathogenic following adoptive transfer into older mice (33), indicating that also the immaturity of the innate immune system contributes to EAE resistance in young animals (31). Compared to young (8-12 week) Biozzi mice that develop RREAE followed by SPEAE, disease induction in 12 month old mice triggered marked progressive EAE from onset. During ageing, thymic involution amplifies autoreactive T-cells and thus the propensity to develop autoimmunity (34). Whether or not the progressive failure of negative selection upon ageing, facilitated by decreased expression of the autoimmune regulator or impaired generation of regulatory T cells, additionally plays a role in the exclusive development of PEAE in old mice requires further study. In addition, the age-related decline in antigen-presenting cell function and myeloid-derived suppressor cells, coupled with the increased expression of MHC-class II antigens and co-stimulatory molecules (31) may explain the resistance against EAE in young mice and the susceptibility to more severe disease in old mice.

That $CD4^+$ cells are more resistant to apoptosis and regulatory T-cell suppression suggests that immunosenescence in MS resembles dysfunctions associated with ageing (35), possibly explaining the higher numbers of T-cells in PEAE in old mice. However, in contrast to aEAE and PEAE, perivascular infiltrates in the CNS declined with disease progression and T cells in the CNS during SPEAE were significantly reduced. Whether this is due to T-cell exhaustion as described in EAE in SJL mice (36) or increased T-cell apoptosis in the CNS is unclear. However, SPEAE continues despite ablation of T cells indicating that disease progression is independent of peripheral T-cell responses (24). Moreover, rather than the manifestation of adaptive immune responses, the SPEAE lesion, like those in PEAE is dominated by activated microglia (25,26) underscoring the role of innate immunity in neurodegenerative diseases. In old mice with PEAE, this may reflect enhanced priming of microglia and an increased expression of proinflammatory cytokines, as have been observed in old mice and Ercc1 mutant mice, a DNA repair-deficient mouse model that displays features of accelerated ageing in multiple tissues including the CNS (37). Thus, atrophy of the thymus as well as alterations in peripheral immune factors are likely to play a concerted role in the now well-recognized phenomenon of 'inflamm-ageing', such as an increased propensity for tissue-damaging inflammatory processes to develop upon ageing (11). Interestingly, in comparison to perivascular infiltrates and T-cell numbers, the numbers of microglia clusters associated with axonal damage and $HSPB5^+$ oligodendrocytes in NAWM and NAGM did not decline with age or during disease progression in RREAE, SPEAE and PEAE. Higher numbers of microglia clusters in the white matter were associated with remission, suggesting that such clusters, like so-called preactive lesions in MS, may perform a regulatory role (38).

Remyelination in the CNS is key to repair and recovery from relapses in MS and although extensive remyelination is observed in MS lesions, it is frequently insufficient to fully restore efficient conduction (39). The causes of incomplete remyelination in MS is unknown but may be a function of age (40) as well as

recurrent episodes of myelin damage. The resistance to EAE of young mice and increased susceptibility of old mice to PEAE may likewise also be a function of the regenerative capability of a youthful CNS. This was recently shown using lysolecithin-induced demyelination in dual-colour reporter mice, by revealing differences in the proliferation, recruitment and differentiation of dorsal and ventral oligodendrocyte progenitors as a factor of age (41). In line with this, heterochronic parabiosis has shown that exposure of aged mice to a youthful systemic milieu stimulates repair following toxin-induced demyelination with lysolecithin (8). Pusic et al. (42) reported this to be the effect of the production of peripheral exosomes that stimulate OPC differentiation and myelination. Whether such factors can prevent or ameliorate SPEAE and PEAE in old mice requires further investigation.

In summary this study supports the notion that ageing as well as recurrent episodes of inflammation in the CNS contribute to experimentally induced progressive neurological disease. While RRMS is characterised by higher disease activity and inflammation in the CNS, pathology studies do not distinguish between PPMS and SPMS. Our observation that three different manifestations of EAE can be induced in the same mouse strain, and are dependent on the age of disease induction, underscores the notion that age and its response to insults plays a major role in the clinical manifestation of autoimmune demyelinating disease. Thus, the models described here not only provide relevant pre-clinical models to develop therapies for progressive forms of MS, but they also underscore the existence of different pathological features of neurodegeneration and progressive disease. Clarification of the differences between the pathological mechanisms that contribute to PEAE, SPEAE and RREAE may be useful to uncover the key factors that contribute to progressive forms of MS.

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Conflicts of Interest

JMvN holds equity in Delta Crystallon BV. The other authors have no conflicts of interest to report.

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Table 1. Antibodies used for immunohistochemistry

Antigen	Species	Isotype	Dilution	Antigen Retrieval	Incubation	Source
Primary antibodies						
IBA-1	Rabbit	IgG	1:10,000	Tris/EDTA	1h	Wako
PLP (plpc1)	Mouse	IgG2a	1:3000	N/A	1h	AbDSerotec
Non-phosphorylated NF-H (SMI-32)	Mouse	IgG1	1:1000	Citrate	1h	BioLegend
HSPB5 (W3/13)	Rabbit	IgG	1:5000	Citrate	O/N	Delta Crystallon BV
CD45R (RA3-6B2)	Rat	IgG2a	1:1500	Citrate	1h	AbDSerotec
CD3 (F7.2.38)	Rabbit	IgG1	1:1000	Citrate	O/N	Dako
Secondary antibodies						
Rabbit IgG-AP	Goat	N/A	1:250	N/A	1h	Southern Biotech
Mouse IgG2a-HRP	Goat	N/A	1:250	N/A	1h	Southern Biotech
Mouse IgG1-HRP	Goat	N/A	1:250	N/A	1h	Southern Biotech
Rabbit EnVision™	Goat	N/A	N/A	N/A	1h	Dako

N/A - not applicable; O/N - overnight

Table 2. Age determines the clinical course of EAE in Biozzi ABH mice

Age mice	N° aEAE[a]	Mortality	EAE score[b]	Day onset	N° remission	N° PEAE
2 weeks	0/11***	0/11	N/A	N/A	N/A	N/A
3 weeks	3/22**	0/16	0.2 ± 0.1**	18.2 ± 2.0	N/A	N/A
8-12 weeks	12/12	0/12	3.9 ± 0.1	12.5 ± 0.3	12/12	0/12
8 months	13/13	1/13*	4.1 ± 0.1	11.2 ± 0.5	10/12	2/12
12 months	10/10	8/10***	4.8 ± 0.2**	14.1 ± 0.4	0/8	8/8

[a]Biozzi mice of different ages were immunised with SCH in CFA and monitored until day 60 where appropriate. [b]max clinical score during the acute EAE. **$p < 0.01$; *** $p < 0.001$ compared to 8- 12 week old mice.

Supplementary Table 1. Clinical History of Mice with EAE

[a]EAE Stage and mouse number	Acute Day onset ([b]score)	Remission 1 Day onset ([c]score)	Relapse 1 Day onset ([b]score)	Remission 2 Day onset ([c]score)	Relapse 2 Day onset ([a]score)	Day Sampling [d]EAE score
Acute (aEAE) m1	14 (4)	N/A	N/A	N/A	N/A	14 (4)
m2	12 (4)	N/A	N/A	N/A	N/A	12 (4)
m3	14 (4)	N/A	N/A	N/A	N/A	14 (4)
m4	12 (4)	N/A	N/A	N/A	N/A	12 (4)
m5	15 (4.5)	N/A	N/A	N/A	N/A	15 (4.5)
m6	15 (4)	N/A	N/A	N/A	N/A	15 (4)
m7	13 (4.5)	N/A	N/A	N/A	N/A	13 (4.5)
m8	20 (4.5)	N/A	N/A	N/A	N/A	20 (4.5)
Remission 1 m1	15 (3.5)	21 (0)	N/A	N/A	N/A	60 (0)
m2	15 (3)	24 (0)	N/A	N/A	N/A	60 (0)
m3	14 (4)	25 (0)	N/A	N/A	N/A	68 (0)
m4	12 (4)	24 (0)	N/A	N/A	N/A	24 (0)
m5	16 (4)	32 (0)	N/A	N/A	N/A	32 (0)
m6	12 (4)	68 (0)	N/A	N/A	N/A	68 (0)
m7	12 (4)	51 (0)	N/A	N/A	N/A	51 (0)
m8	14 (4)	59 (0)	N/A	N/A	N/A	59 (0)
Relapse 1 m1	14 (4)	31 (0)	41 (4.5)	N/A	N/A	48 (4.5)
m2	15 (4)	29 (0)	34 (4)	N/A	N/A	42 (4)
m3	15 (4)	24 (0)	44 (2.5)	N/A	N/A	60 (1.5)
m4	15 (4)	24 (0)	33 (4.5)	N/A	N/A	42 (4.5)
m5	14 (4)	30 (0)	32 (4)	N/A	N/A	43 (4)
m6	11 (4)	31 (0)	41 (4.5)	N/A	N/A	38 (4)
m7	11 (4)	27 (0.5)	30 (4)	N/A	N/A	38 (4.5)
m8	11 (4)	23 (0)	37 (4)	N/A	N/A	51 (4.5)
Remission 2 m1	15 (4)	22 (0)	40 (4.5)	52 (0)	N/A	60 (0)
m2	14 (4)	24 (0)	36 (2)	46 (0)	N/A	69 (0)
m3	12 (4)	20 (0)	38 (3.5)	69 (0)	N/A	71 (0)
m4	13 (4)	21 (0)	25 (2.5)	32 (0)	N/A	32 (0)
m5	12 (4.5)	27 (0)	32 (3.5)	36 (0)	N/A	47 (0)
m6	14 (4)	29 (0)	44 (2.5)	52 (0)	N/A	69 (0)
m7	14 (4)	24 (0)	36 (2)	46 (0)	N/A	69 (0)
SPEAE m1	15 (4)	21 (0)	37 (3.5)	49 (0.5)	62 (3.5)	69 (3.5)
m2	16 (4)	22 (0)	38 (4)	51 (1.0)	59 (4)	66 (4)
m3	14 (4)	25 (0.5)	40 (4.5)	63 (0.5)	72 (4)	78 (4)
m4	17 (4)	23 (0)	25 (2.5)	52 (0.5)	68 (3.5)	82 (3.5)
m5	13 (4)	28 (0.5)	37 (4)	48 (2.5)	71 (3.5)	101 (3.5)
m6	11 (4)	26 (0)	29 (4)	61 (1.5)	92 4.0)	98 (4)
m7	17 (4)	24 (0.5)	39 (4)	52 (2.5)	79 (3.5)	115 (3.5)
m8	16 (4)	23 (0.5)	38 (4)	50 (2.5)	89 (3.5)	115 (3.5)
PEAE 12 months m1	15 (4.5)	N/A	N/A	N/A	N/A	19 (4.5)
m2	13 (5)	N/A	N/A	N/A	N/A	20 (5)
m3	15 (5)	N/A	N/A	N/A	N/A	19 (5)
m4	13 (4.5)	N/A	N/A	N/A	N/A	16 (4.5)
m5	16 (4.5)	N/A	N/A	N/A	N/A	22 (4.5)
m6	15 (5)	N/A	N/A	N/A	N/A	16 (5)
m7	17 (4.5)	N/A	N/A	N/A	N/A	19 (4.5)
m8	12 (4.5)	N/A	N/A	N/A	N/A	19 (4.5)

[a]Biozzi mice (m) were immunised with SCH in CFA (see materials and methods) and sampled at different stages during EAE. [b]maximal clinical score during EAE; [c]minimum clinical score during remission; [d]mice with SPEAE had exhibited aEAE and 2 relapses and were sampled when they had exhibited grade 4 for 7 d or at stated date when they exhibited grade 3.5.

Figure Legends

Figure 1. Ageing and EAE augments IBA-1+ microglia/macrophages in mice

Immunohistochemistry was used to detect IBA-1+ cells in the spinal cord of young and old control mice (upper panels) and young and old mice with EAE (lower panels). Numbers of IBA-1^+ microglia/macrophages in the spinal cord are higher in old mice as compared to young mice (A-D, E). During aEAE in young mice similar numbers of IBA-1^+ cells are observed in white and grey matter (F, G, J) while an increase is observed in the grey matter of old mice with PEAE (G, I, J). Inserts show the morphology of a single IBA-1+ cells. *P<0.05; *** P<0.001. Mean ± SEM (n = 8). Scale bar= 50 µm

Figure 2. PEAE but not SPEAE is associated with increased numbers of $CD3^+$ T cells

The expression of CD3 (A, B) and B220 (C, D) was evaluated in the white and grey matter of the spinal cord of young mice with aEAE and SPEAE, and in old mice with PEAE. CD3 expression was increased in the white (A) and grey (B) matter during PEAE as compared to aEAE or SPEAE. B220 expression was lower than that of CD3 (note scale of X-axis in C and D compared to A and B). $B220^+$ cells in white matter (C) and grey matter (D) during aEAE, PEAE and SPEAE reveal a significant increase in grey matter during PEAE. *P<0.05; **P<0.01; *** P<0.001. Mean ± SEM (n = 6).

Figure 3. IBA-1^+ cells in PEAE are associated with severe axonal damage

Representative images of spinal cords from young and old control (CON) mice, or mice during aEAE and PEAE (n = 6 group) show IBA-1^+ cells and their association with axonal damage as determined by SMI-32 expression. No axonal damage was observed in control mice (A, B). Axonal damage was observed in young mice during aEAE (C, arrow; insert), and was associated with IBA-1^+ cells (C, arrowhead). Old mice with PEAE (D) developed severe axonal damage (D, insert) associated with increased numbers of IBA-1^+ cells. Scale bar = 25 µm.

Figure 4. IBA-1^+ cells in perivascular clusters and microglia clusters

PVC were identified as clusters of 4 or more IBA-1+ cells closely associated with blood vessels (bv) in normal appearing myelin as determined by PLP expression (brown) in white matter (WM; A) or grey matter (GM; B). MC were identified as 4 or more IBA-1+ microglia in NAWM (C) or NAGM (D) that were not associated with endothelial cells or erythrocytes. Scale bar = 25 µm.

Figure 5. Numbers of perivascular IBA-1+ clusters but not of microglia clusters decline during progression of EAE

Numbers of PVC (A-D) and MC (E-H) were quantified in the spinal cord and brain during PEAE in old mice (hatched bars) and during RREAE and SPEAE in young mice. Compared to RREAE (white bars) or PEAE mice the numbers of PVC in the spinal cord (A) and brain (B) were significantly reduced in SPEAE. This difference was more pronounced in spinal cord white matter (C) than grey matter (D).

In contrast, numbers of MC in spinal cord (E) and brain (F), both in the white (G) and grey (H) matter, revealed higher numbers of MC during the second remission. Mean SEM. * P<0.05; ** P<0.005; *** P<0.001 (n = 8 per group).

Figure 6. Microglia clusters during EAE are associated with axonal damage and HspB5$^+$ oligodendrocytes but not with CD3$^+$ or B220$^+$ lymphocytes

Clusters of IBA-1$^+$ cells in the absence of (A) or in close association with NF-H$^+$ axons (arrow in B) and HspB5$^+$ oligodendrocytes (arrow in C) during EAE. Such clusters were not associated with CD3$^+$ T cells (D) [E is a zoomed-in picture of the box in D], or B220$^+$ B cells (arrow in F). Scale bar = 25 μm.

Legend for Supplementary Figure 1

Numbers of IBA-1+ microglia/macrophages as represented by the number of positive pixels (see materials and methods for description) in the spinal cord are higher in the white (WM) and grey (GM) matter of aEAE in young mice and PEAE in old mice as compared to control aged matched mice *** P<0.001. Mean ± SEM (n = 8).

Figure 1

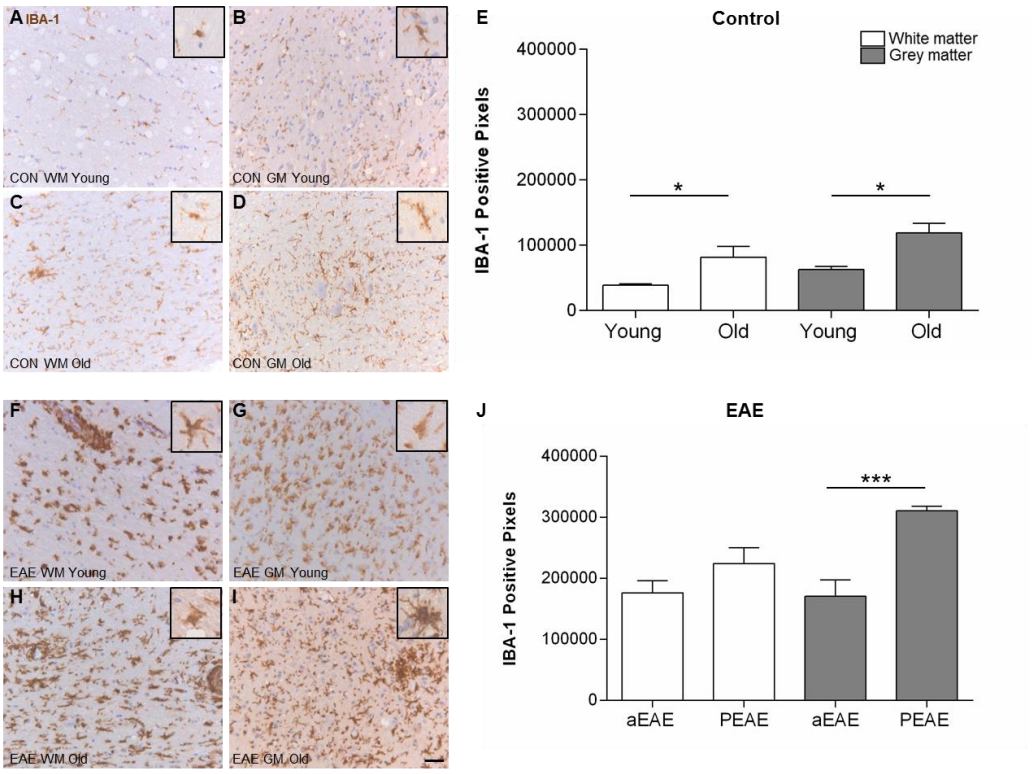


Figure 2

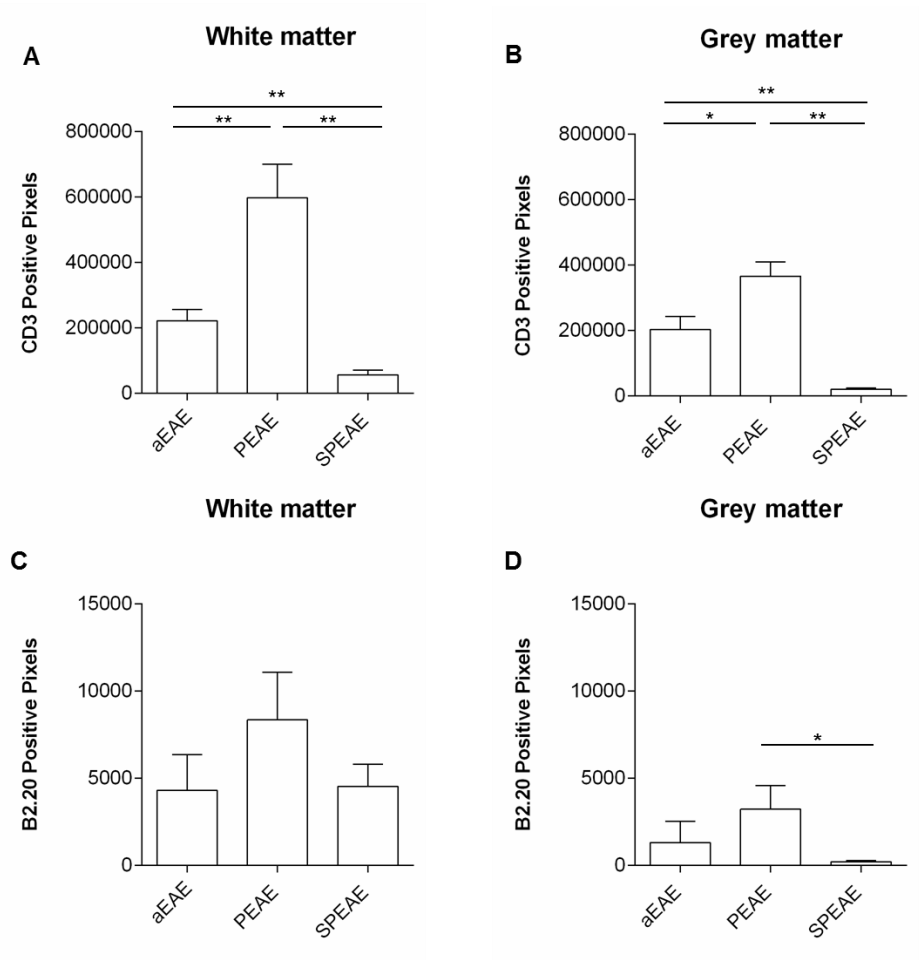


Figure 3

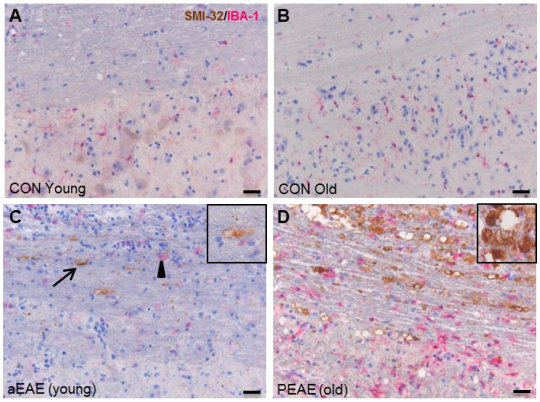


Figure 4

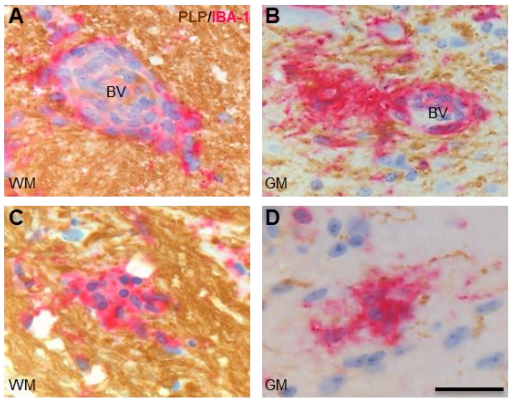


Figure 5

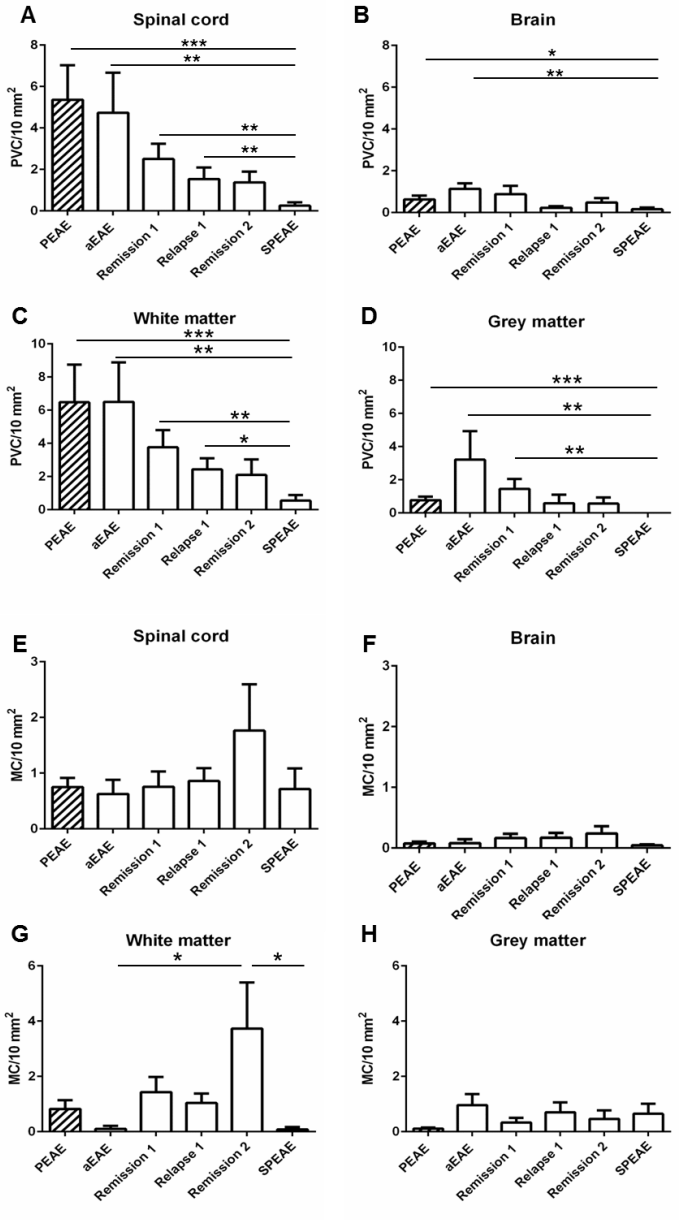
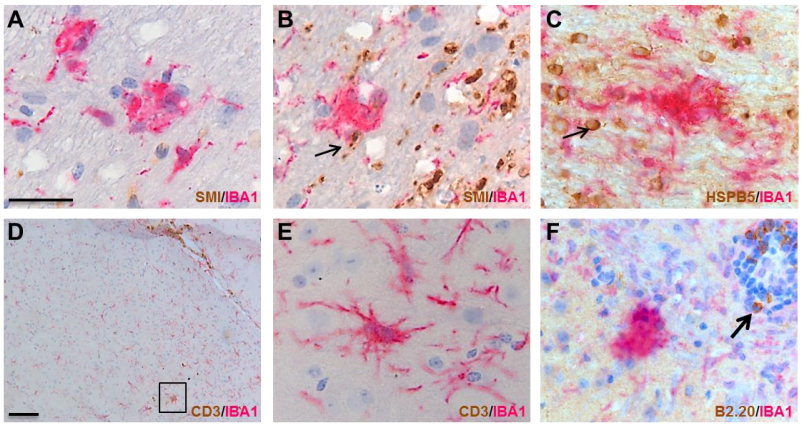


Figure 6



Suppl Figure 1