



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

IL-10-producing, ST2-expressing Foxp3(+) T cells in multiple sclerosis brain lesions

Citation for published version:

Zandee, SEJ, O'Connor, RA, Mair, I, Leech, MD, Williams, A & Anderton, SM 2017, 'IL-10-producing, ST2-expressing Foxp3(+) T cells in multiple sclerosis brain lesions', *Immunology & Cell Biology*.
<https://doi.org/10.1038/icb.2017.3>

Digital Object Identifier (DOI):

[10.1038/icb.2017.3](https://doi.org/10.1038/icb.2017.3)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Immunology & Cell Biology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 **IL-10-producing, ST2-expressing Foxp3⁺ T cells in multiple sclerosis brain lesions**

2 Running title: CD4⁺Foxp3⁺ cells in Multiple Sclerosis lesions.

3 **Stephanie Elizabeth Johanna Zandee¹, Richard Anthony O'Connor¹, Iris Mair¹, Melanie Dawn**
4 **Leech, Anna Williams² and Stephen Mark Anderton¹**

5 **Affiliations:**

6 ¹ MRC Centre for Inflammation Research, The Edinburgh Centre for Multiple Sclerosis Research and
7 Centre for Immunity Infection and Evolution, University of Edinburgh, UK.

8 ² MRC Centre for Regenerative Medicine and The Edinburgh Centre for Multiple Sclerosis Research,
9 University of Edinburgh, UK.

10

11 **Correspondence:**

12 **Stephen M Anderton**

13 University of Edinburgh, Centre for Inflammation Research, Queen's Medical Research Institute
14 47 Little France Crescent, Edinburgh EH16 4TJ United Kingdom

15 steve.anderton@ed.ac.uk

16 Phone: +44 131 242 6589

17 FAX: +44-131-242 6682

18

19 Conflict of interest statement: The authors have declared that no conflict of interest exists.

20

21 This work was supported by grants from the UK Medical Research Council, The Dutch MS Research
22 Foundation and the Scottish Chief Scientist Office.

23

24 **Abstract**

25 CD4⁺Foxp3⁺ T regulatory (Treg) cells provide a key defence against inflammatory disease, but also have
26 an ability to produce pro-inflammatory cytokines. The evidence for these two possibilities in multiple
27 sclerosis (MS) is controversial. However, this has largely been based on studies of circulating Treg cells
28 derived from peripheral blood, rather than the central nervous system. We show that Foxp3⁺ cells in the
29 brains of MS patients predominantly produce IL-10 and show high expression of the IL-33 receptor ST2
30 (associated with potent Treg function), indicating that Treg in the inflamed brain maintain their
31 suppressive function.

32

33 **Main Text**

34 CD4⁺Foxp3⁺ Treg cells control immune responses in inflamed tissues as well as secondary lymphoid
35 organs^{1, 2}. Treg cells isolated from the peripheral blood of MS patients are reported to show reduced
36 suppressive function, but not reduced frequencies³⁻⁵. Treg cells can “trans-differentiate” to a pro-
37 inflammatory function, producing IFN- γ or IL-17, when placed in conducive experimental conditions⁶⁻⁸.
38 Peripheral blood Treg cells from MS patients were reported to display this ability, producing IFN- γ *in*
39 *vitro* under the influence of IL-12⁹. The major drawback of such studies is that, out of necessity, only the
40 peripheral blood of MS patients can be sampled and not the central nervous system (CNS) itself. Tissue
41 inflammation can stabilize, rather than diminish Treg suppressive function. We reported that the
42 accumulation of highly activated and suppressive, IL-10-producing Treg cells within the CNS is
43 necessary for the natural resolution of experimental autoimmune encephalomyelitis (EAE), a mouse
44 model of MS^{10, 11}. In addition, these CNS Treg cells resisted conversion to pro-inflammatory function *in*
45 *vitro*¹². Here, we sought to understand the distribution of Treg in human MS lesions and to gather
46 evidence for suppressive, or pro-inflammatory roles for these cells.

47

48 **Results and Discussion**

49 Immunohistochemistry identified the presence of CD4⁺Foxp3⁺ T cells in post mortem brain tissue of 7/11
50 secondary progressive MS (SPMS) patients (Fig. 1a), with none found in control brain tissue. MS
51 samples that did or did not contain CD4⁺Foxp3⁺ cells could not be distinguished based on patient gender,
52 age, duration of disease, or time to post mortem processing (summarised in Supp Table 1). CD4⁺Foxp3⁺
53 cells were distributed at similar frequencies across different white matter lesion types (9/10 active lesions,
54 3/7 chronic active borders, 3/7 chronic active centres, 9/17 chronic inactive lesions), but not in
55 remyelinating lesions (Fig. 1b). Thus, Treg presence in MS lesions appears to be associated with the
56 presence of an inflammatory infiltrate (not found in remyelinating lesions). This is consistent with our
57 previous EAE data showing that Treg numbers in the CNS decline markedly, in-line with the
58 inflammatory infiltrate, as the disease resolves^{10,11}. Where present, the frequencies of CD4⁺ cells that

59 were Foxp3⁺ ranged between 10-30% (Fig. 1c), which represents an enrichment over the expected
60 frequencies of these cells amongst CD4⁺ T cells in human peripheral blood (1-3% in healthy controls and
61 MS patients) and in cerebrospinal fluid (3-4% in MS)^{3, 4, 13}.

62 **Although the presence of CD4⁺IL-17⁺ T cells has been reported before¹⁴, no analogous analysis**
63 **has been made of cytokine production by Foxp3⁺ cells in MS lesions.** Two-colour immunohistochemistry
64 identified co-expression of Foxp3 with IL-10, IL-17, IFN- γ , or GM-CSF in active and chronic lesions
65 (Fig. 2a). Approximately 50% of Foxp3⁺ cells stained positive for IL-10 (Fig. 2b). Lower frequencies of
66 Foxp3⁺ cells stained positive for pro-inflammatory cytokines. IL-10 was the dominant cytokine produced
67 by Foxp3⁺ cells (>60%) in active lesions and the borders of chronic active lesions (Fig. 2b). This was less
68 evident in chronic inactive lesions and in the centres of chronic active lesions, where Foxp3⁺ cells showed
69 no enrichment in IL-10 over other cytokines. In contrast to IL-10, TNF- α staining in Foxp3⁺ cells only
70 became evident in chronic inactive lesions. Frequencies of Foxp3⁺ cells staining for IFN- γ , IL-17, or GM-
71 CSF were low in all active and chronic lesion types. We conclude that the predominant cytokine produced
72 by Foxp3⁺ cells within the brains of SPMS patients is IL-10. This is entirely consistent with our previous
73 observations of Treg in the CNS of mice with EAE¹⁰⁻¹² and indicates that, in MS, Treg that infiltrate the
74 lesions are in suppressive rather than pro-inflammatory mode.

75 As CD4⁺Foxp3⁺ cells composed only a minor fraction of infiltrating cells within lesions, their
76 contribution to the overall cytokine⁺ cells remained modest, even for IL-10. We compared the frequencies
77 of CD4⁺Foxp3⁻ or CD4⁺Foxp3⁺ cells in **all** lesions, with the overall levels of cytokine⁺ cells in those
78 lesions. CD4⁺Foxp3⁻ frequencies did not correlate with any cytokine (Fig. 3a). Nor did CD4⁺Foxp3⁺ cells
79 correlate with IFN- γ , GM-CSF or IL-17. However, CD4⁺Foxp3⁺ frequencies correlated with the
80 frequencies of total IL-10⁺ cells and total TNF- α ⁺ cells (Fig. 3b).

81 Elegant murine studies have shown that IL-10 signalling in Treg cells is required for their own
82 IL-10 expression and subsequent suppressive function¹⁵. Therefore it is plausible that, in addition to
83 contributing to the IL-10 pool, IL-10⁺ Treg cells are specifically attracted to, expanded in, or maintained

84 in lesions with high IL-10 levels. TNF- α -blockade is a potent therapeutic option for several human
85 inflammatory diseases such as rheumatoid arthritis, Crohn's disease and psoriasis^{16, 17}, but not MS¹⁸.
86 Studies on how TNF- α -blockade effects the Treg populations have led to conflicting results. TNF- α
87 blockers have been reported to increase the number or function of Treg cells in RA and Crohn's^{19, 20}.
88 However, it has also been shown to inhibit suppressive function of Treg cells through down-regulation of
89 Foxp3 in RA patients²¹. Recent studies indicate TNF- α signals selectively through TNFR2 in Treg cells²²,
90 ²³. This suggests that Treg cells might require TNF- α for their suppressive function and provides a
91 plausible explanation for the positive correlation between Foxp3⁺ cells and TNF- α ⁺ cells that we see.

92 Expression of the IL-33 receptor, ST2, has been associated with potent Treg function in murine
93 models²⁴⁻²⁶. Indeed, we found ST2 to be particularly enriched in CNS Treg in EAE (Fig. 4a). IL-33 is
94 highly expressed in the CNS in both EAE and MS (Fig. 4b)^{27, 28}. Dual immunofluorescence identified the
95 presence of Foxp3⁺ST2⁺ cells in MS brains (Fig. 4c). In particular, ~60% of Foxp3⁺ cells in active lesions
96 were ST2⁺, whilst its expression was almost absent in Foxp3⁺ cells in chronic lesions (Fig. 4d). High
97 expression of both IL-10 (Fig. 2b) and ST2 (Fig. 4d) by Foxp3⁺ cells in active lesions suggests that their
98 suppressive potency should be greatest in these lesions and that this might wane in more chronic lesions.
99 A recent study from Miron et al²⁹ demonstrated high numbers of M2 macrophages, also particularly in
100 active lesions, of the same brain tissue studied here. This is interesting for two reasons. Firstly, IL-10
101 (perhaps originating from Treg cells) can promote the M2 phenotype, which is thought to contribute to
102 remyelination by inducing oligodendrocyte differentiation. Secondly, a study of experimental cerebral
103 malaria recently reported that IL-33 is protective by coordinating both Treg and M2 activity (the latter via
104 expansion of type-2 innate lymphoid cells which release M2-promoting cytokines)³⁰. Whether such a
105 coordinated response is protective in CNS autoimmune inflammation, and whether there are viable
106 therapeutic approaches that can boost the numbers and/or sustain the function of these cells, should be
107 fruitful avenues for exploration.

108

110 **Methods**

111 *Human Tissue specimens*

112 Post-mortem tissue from SPMS patients and control individuals who died of **non-neurological causes**
113 were obtained via a UK prospective donor scheme with full ethical approval and informed consent from
114 the UK Multiple Sclerosis Tissue Bank (MREC/02/2/39)(Supplementary information Table 1). Snap
115 frozen unfixed tissue blocks from 11 SPMS patients (a total of 16 blocks containing 10 active lesions, 7
116 chronic active lesions, 17 chronic inactive lesions and 12 remyelinating lesions) and 4 control blocks were
117 analysed. Lesions were classified as active, chronic active, chronic inactive and remyelinating according
118 to the International Classification of Neurological Diseases (www.icdns.org) using **Luxol Fast Blue –**
119 **Cresyl Violet staining and Oil Red O staining.**

120 *Immunohistochemistry of T cell subsets*

121 10 µM sections were fixed in 4% PFA (Fisher Scientific, Waltham, USA) and subsequently delipidised in
122 70% ice-cold ethanol. Antigens were retrieved using heating in acid citric buffer (Vector, Burlingame,
123 USA). Sections were incubated with anti-Foxp3 (ab10563, **rabbit**, Abcam, Cambridge, UK) overnight at
124 4°C. Subsequently the sections were incubated with anti-CD4 (M7310, **mouse**, Dako, Glostrup, Denmark)
125 for 30 minutes at room temperature. An EnVision G2 Doublestain System, **Rabbit/Mouse** kit (Dako) was
126 used for detection as per manufacturer's instructions, with exception of the use of an Vector Blue
127 Alkaline Phosphatase Substrate Kit III (Vector) to develop the signal. Sections were mounted in aqueous
128 permafluor medium (Thermo Scientific, Waltham, USA). Primary antibodies were omitted to check for
129 non-specific binding of polymers. **Rabbit IgG (ab27478, Abcam) or Mouse IgG1 isotype control (X0931,**
130 **Dako) were used to control for non-specific binding of the primary antibodies. All IHC experiments were**
131 **performed in triplicate.**

132

133

134 *Immunohistochemistry of cytokines*

135 For double staining of Foxp3 and cytokines, combinations of antibodies against TNF- α , IFN- γ , IL-17,
136 GM-CSF or IL-10 (AF-210-NA, AF-285-NA, AF-317-NA, AF-215-NA, AF-217-NA, all goat, R&D
137 systems, Abingdon, UK) with anti-Foxp3 (rabbit, Abcam) were used. For single IL-33 staining a goat
138 anti-IL-33 antibody (AF3625, R&D systems) was used. Briefly, frozen brain sections were fixed in 4%
139 PFA (Fisher Scientific), followed by antigen retrieval as described above. Endogenous peroxidase was
140 blocked with 3% H₂O₂ in dH₂O (Fisher Scientific), followed by blocking of biotin for 15 minutes
141 (Vector). Sections were incubated with 10% horse serum in PBS (Biosera, Boussens, France) and Fc
142 Receptor Blocking Solution was added (Human TruStain FcX Biologend, London, UK). Primary
143 antibodies were added overnight at 4°C. Cytokines were detected with donkey anti-goat-biotin (ab6578,
144 Abcam) followed by streptavidin-alkaline phosphatase (SA-5100, Vector) and visualized with the Vector
145 Blue Alkaline Phosphatase Substrate Kit III (Vector). Slides were blocked with 10% goat serum in PBS
146 (Biosera). Anti-Foxp3 (rabbit) was detected with an anti-rabbit polymer-HRP (Dako) and developed with
147 DAB substrate (Dako). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Life
148 Technologies, Carlsbad, USA), and mounted in aqueous permafluor medium (Thermo Scientific).
149 Secondary antibodies/polymers alone, or normal goat IgG (AB-108-C, R&D Systems) and rabbit IgG
150 (Abcam) were used to control for non-specific binding.

151

152 *Immunofluorescent staining of Foxp3 and ST2*

153 Sections were air dried overnight, fixed in ice-cold acetone (VWR) and air dried for 30 minutes.
154 Endogenous peroxidase and biotin were blocked as described above. Sections were blocked with 10%
155 goat serum (Biosera) in PBS and incubated with rabbit anti-Foxp3 (Abcam) overnight at 4°C. Foxp3
156 antibody was detected with a goat-anti-rabbit-biotinylated antibody (BA-1000, Vector), followed by
157 incubation with a streptavidin-coupled horseradish peroxidase (SA-5004, Vector). Tyramide-Cy3 (Perkin-
158 Elmer, Waltham, USA) was applied for 10 minutes to visualize the staining and ST2L FITC antibody
159 (MdBioProducts, Zürich, Switzerland) was incubated overnight at 4°C. Sections were counterstained with

160 DAPI (Life Technologies) and mounted in aqueous Permafluor medium (Thermo Scientific). **Mouse**
161 **IgG1 FITC (1053002F, MdBioProducts), rabbit IgG (Abcam), or** secondary antibodies/polymers alone
162 were used to control for non-specific binding. Only lesions with Foxp3⁺ cells were analysed.

163

164 *EAE induction*

165 C57BL/6 mice were bred under specific pathogen free conditions at the University of Edinburgh. All
166 experiments were approved by the University of Edinburgh Ethical Review Committee and were
167 performed in accordance with UK legislation. **Female** mice were used between 6-12 weeks old (**n = 7**).
168 EAE was induced by administration 100µg of MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK,
169 Cambridge Research Biochemicals, Teesside, UK), emulsified in complete Freund's adjuvant containing
170 200µg of heat-inactivated *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich), with a total volume of
171 100µl injected subcutaneously into the hind legs. On the same day and 48 hours later, 200ng of pertussis
172 toxin (Health Protection Agency, Dorset, UK) was given in 0.5ml of PBS intraperitoneally. Clinical signs
173 of EAE were assessed daily with the following scoring system: 0, no signs; 1, flaccid tail; 2, impaired
174 righting reflex and/or gait; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; 5, hindlimb paralysis
175 with partial forelimb paralysis; 6, moribund or dead.

176

177 *Isolation of CNS mononuclear cells and flow cytometry*

178 Mice were sacrificed at d16 (when Treg were evident in the CNS) by CO₂ asphyxiation and perfused with
179 PBS. Brains and spinal cords were removed, mechanically disrupted and digested in RPMI containing 7.5
180 mg/ml collagenase type 4 (Lorne Laboratories, Reading, UK) and 2.5 mg/ml DNase I (Sigma-Aldrich)
181 for 30 minutes at 37°C. Mononuclear cells were isolated from the interface of a 30%:70% discontinuous
182 Percoll gradient (GE healthcare, Uppsala, Sweden) after centrifugation at 530xg for 20 minutes. Cells
183 were stained using the following antibodies: anti-CD4 brilliant violet 650 (Biolegend), anti-Foxp3 eFluor
184 450 (**eBioscience, San Diego, USA**), anti-ST2 FITC (MdBioscience).

185

186 *Data acquisition*

187 Immunohistochemistry samples were analysed using an Olympus AX70 microscope (Olympus
188 Corporation, Tokyo, Japan). The number of cells was always quantified in the whole lesion and expressed
189 as cells per mm² within different lesion types. The total number of nuclei was also documented. An
190 AxioScan.Z1 slide scanner (Zeiss, Cambridge, UK) was used to acquire fluorescent images and Zen Blue
191 software (Zeiss) used to process the fluorescent images. Experiments were repeated 2-3 times and
192 analysed blinded. Flow cytometric data was acquired using a Becton Dickinson (BD, Franklin Lakes,
193 USA) LSRFortessa II and analysed using FlowJo software (Tree Star version 3.2.1, Ashland, USA).

194

195 *Statistical analysis*

196 Where data were unevenly distributed, log transformations and statistical analysis was performed using a
197 linear mixed model. This model accounts for random effects such as having different numbers of tissue
198 blocks from each patient. In case of multiple testing, significant values were corrected with the
199 Bonferroni test. When random effects were found to be non-significant, simplified statistical tests such as
200 a Mann-Whitney-U test or a Kruskal-Wallis test were used. In case of multiple testing using a Kruskal-
201 Wallis test, significant values were corrected with Dunn's multiple comparison test. **Correlations were**
202 **performed using Spearman rank correlation tests. Lesions were not subdivided into pathological types,**
203 **thereby allowing sufficient numbers for analysis.** SPSS version 19 (IBM, New York, USA) statistical
204 software and Prism version 5.04 (Graphpad, La Jolla, USA) software were used to perform the
205 calculations. Data are presented as mean ± SEM. Significant differences are denoted as * p<0.05, **
206 p<0.01 and *** p<0.001.

207

208 **Acknowledgements:** We thank the UK Multiple Sclerosis Tissue Bank for providing human brain tissue,
209 F. Roncaroli (Imperial College London) for neuropathological diagnosis, R. Nicholas (Imperial College
210 London) for providing clinical histories, Mr. D.M. Mole for collaboration in developing several
211 immunohistochemistry techniques, Ms. A. Boyd for technical assistance, Dr. M. Chase-Topping for

212 statistical advice and staff of Flow Cytometry and Histology/Imaging Facilities. This work was supported
213 by the Scottish Chief Scientist Office (ETM/163), the Dutch MS Foundation (r12-1MS) and the UK
214 Medical Research Council (G0801924).

215

216 **Conflicts of interest:** The authors have no conflicting interests to declare.

217

218 Supplementary information is available at the Immunology and Cell Biology website.

219

220 **References**

221 1. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice
222 by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a
223 T cell subset as a possible cause of autoimmune disease. *J Exp Med* 1985; **161**: 72-87.

224 2. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune
225 dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat*
226 *Genet* 2001; **27**: 20-21.

227 3. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+
228 regulatory T cells in patients with multiple sclerosis. *J Exp Med*. 2004; **199**: 971-979.

229 4. Haas J, Hug A, Viehovec A, Fritzsching B, Falk CS, Filser A, et al. Reduced suppressive effect of
230 CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in
231 patients with multiple sclerosis. *Eur J Immunol* 2005; **35**: 3343-3352.

232 5. Anderton SM, Liblau RS. Regulatory T cells in the control of inflammatory demyelinating diseases of the
233 central nervous system. *Curr Opin Neurol* 2008; **21**: 248-254.

234 6. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or
235 are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 2007; **178**: 6725-6729.

236 7. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of
237 the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* 2009; **10**:
238 1000-1007.

239 8. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing
240 FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci USA* 2009; **106**: 4793-4798.

241 9. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+
242 regulatory T cells in human autoimmune disease. *Nat Med* 2011; **17**: 673-675.

243 10. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune
244 encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol*
245 2005; **175**: 3025-3032.

246 11. O'Connor RA, Malpass KH, Anderton SM. The inflamed central nervous system drives the activation and
247 rapid proliferation of Foxp3+ regulatory T cells. *J Immunol* 2007; **179**: 958-966.

248 12. O'Connor RA, Floess S, Huehn J, Jones SA, Anderton SM. Foxp3(+) Treg cells in the inflamed CNS are
249 insensitive to IL-6-driven IL-17 production. *Eur J Immunol*. 2012; **42**: 1174-1179.

250 13. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, Wiendl H. Increased frequency of CD4+ CD25+
251 regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clin Exp Immunol*
252 2007; **147**: 412-418.

253 14. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in
254 central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J*
255 *Pathol* 2008; **172**: 146-155.

256 15. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in
257 regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 2011; **34**: 566-578.

258 16. Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev*
259 *Immunol* 2001; **19**: 163-196.

260 17. MacDonald TT, Hutchings P, Choy MY, Murch S, Cooke A. Tumour necrosis factor-alpha and interferon-
261 gamma production measured at the single cell level in normal and inflamed human intestine. *Clin Exp Immunol*
262 1990; **81**: 301-305.

263 18. van Oosten BW, Barkhof F, Truyen L, Boringa JB, Bertelsmann FW, von Blomberg BME, et al. Increased
264 MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor
265 necrosis factor antibody cA2. *Neurology* 1996; **47**: 1531-1534.

266 19. Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell
267 population in patients with rheumatoid arthritis via TGF-beta. *J Exp Med* 2007; **204**: 33-39.

268 20. Ricciardelli I, Lindley KJ, Londei M, Quarantino S. Anti tumour necrosis-alpha therapy increases the
269 number of FOXP3 regulatory T cells in children affected by Crohn's disease. *Immunology* 2008; **125**: 178-183.

270 21. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates
271 the function of human CD4+CD25hi T-regulatory cells. *Blood* 2006; **108**: 253-261.

272 22. Chen X, Baumel M, Mannel DN, Howard OM, Oppenheim JJ. Interaction of TNF with TNF receptor type
273 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol* 2007; **179**: 154-161.

- 274 23. Chen X, Wu X, Zhou Q, Howard OM, Netea MG, Oppenheim JJ. TNFR2 is critical for the stabilization of
275 the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. *J Immunol* 2013;**190**:1076-1084.
- 276 24. Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33
277 promotes regulatory T-cell function in the intestine. *Nature* 2014; **513**: 564-568.
- 278 25. Matta BM, Lott JM, Mathews LR, Liu Q, Rosborough BR, Blazar BR, et al. IL-33 is an unconventional
279 Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *J*
280 *Immunol* 2014; **193**: 4010-4020.
- 281 26. Siede J, Frohlich A, Datsi A, Hegazy AN, Varga DV, Holecska V, et al. IL-33 Receptor-Expressing
282 Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and
283 TGFbeta Release. *PLoS One* 2016; **11**: e0161507.
- 284 27. Christophi GP, Gruber RC, Panos M, Christophi RL, Jubelt B, Massa PT. Interleukin-33 upregulation in
285 peripheral leukocytes and CNS of multiple sclerosis patients. *Clin Immunol* 2012; **142**: 308-319.
- 286 28. Jiang HR, Milovanovic M, Allan D, Niedbala W, Besnard AG, Fukada SY, et al. IL-33 attenuates EAE by
287 suppressing IL-17 and IFN-gamma production and inducing alternatively activated macrophages. *Eur J Immunol*
288 2012; **42**: 1804-1814.
- 289 29. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages
290 drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* 2013; **16**: 1211-1218.
- 291 30. Besnard AG, Guabiraba R, Niedbala W, Palomo J, Reverchon F, Shaw TN, et al. IL-33-mediated
292 protection against experimental cerebral malaria is linked to induction of type 2 innate lymphoid cells, M2
293 macrophages and regulatory T cells. *PLoS Pathog* 2015; **11**: e1004607.
- 294

295 **Figure legends**

296 **Figure 1. CD4⁺Foxp3⁺ T cell are enriched within MS lesions.**

297 (a) Representative images from different MS cases (A = active, CA = chronic active and CI = chronic
298 inactive lesions) of immunohistochemistry for CD4 (blue) and Foxp3 (brown). No staining was observed
299 using isotype controls or secondary antibodies alone. Scale bars 20 μ m. Accompanying images show
300 lesions (LFB = Luxol Fast Blue – Cresyl Violet). Dotted line represents lesion border. Black boxes
301 delineate the areas CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ cells were pictured. Scale bars 200 μ m. (b) Densities of
302 CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ cells in the indicated SPMS lesion types. (c) Frequencies of CD4⁺ cells that
303 were Foxp3⁺ in the indicated SPMS lesion types. Graphs show means \pm SEM. Kruskal-Wallis tests with
304 Dunn's multiple comparison correction were used. * p<0.05, ** p<0.01. 10 active lesions, 7 chronic
305 active lesions, 17 chronic inactive lesions and 12 remyelinating lesions were studied.

306

307 **Figure 2. Foxp3⁺ cells predominantly produce IL-10 in MS lesions.**

308 (a) Representative images of immunohistochemistry for individual cytokines (blue) and Foxp3 (brown).
309 No staining was observed using isotype controls or secondary antibodies alone. Scale bars 20 μ m. (b)
310 Frequencies of Foxp3⁺ cells co-staining for individual cytokines in the indicated SPMS lesion types.
311 Graphs show means \pm SEM. A Kruskal-Wallis test with Dunn's multiple comparison correction was used.
312 ** p<0.01. 5 active lesions, 3 chronic active lesions and 8 chronic inactive lesions were studied.

313

314 **Figure 3. Frequencies of CD4⁺Foxp3⁺ cells correlate with IL-10 and TNF- α levels in MS lesions**

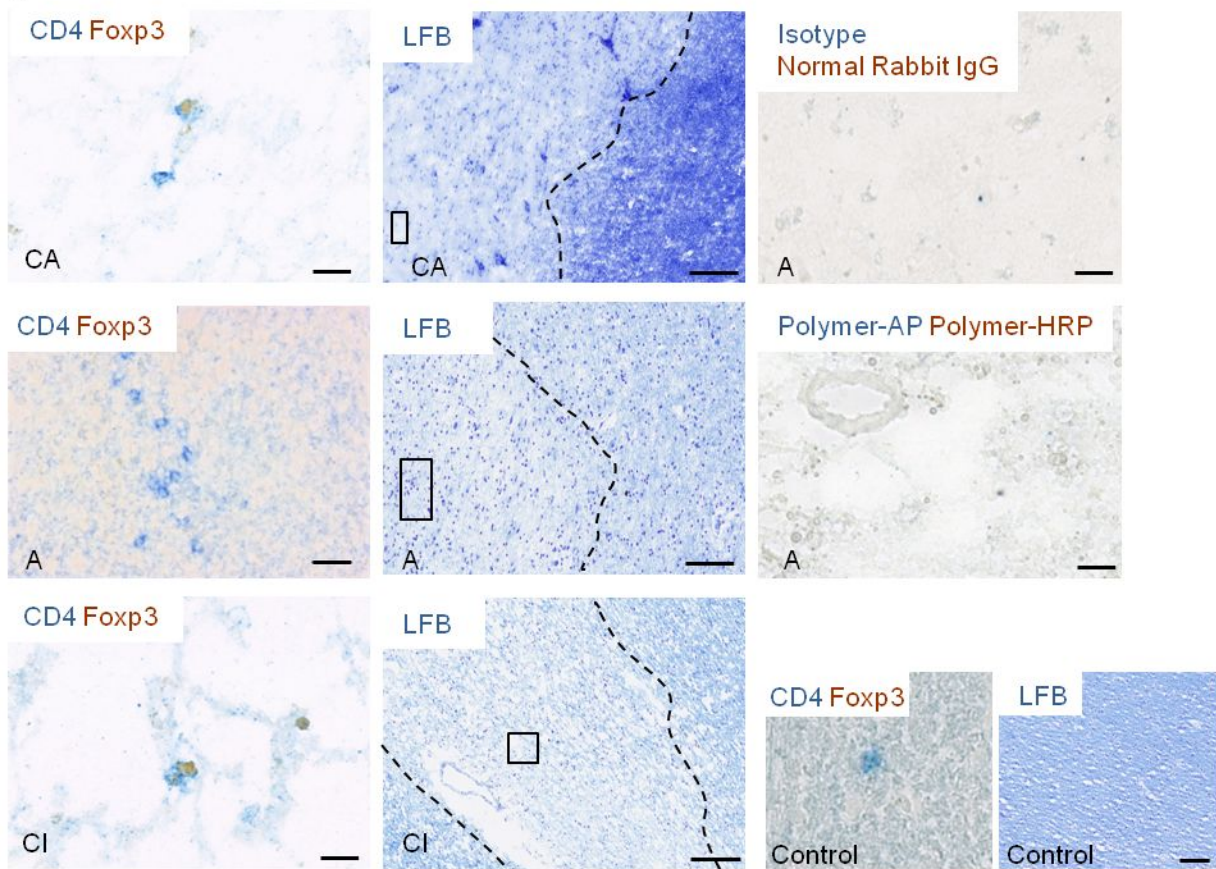
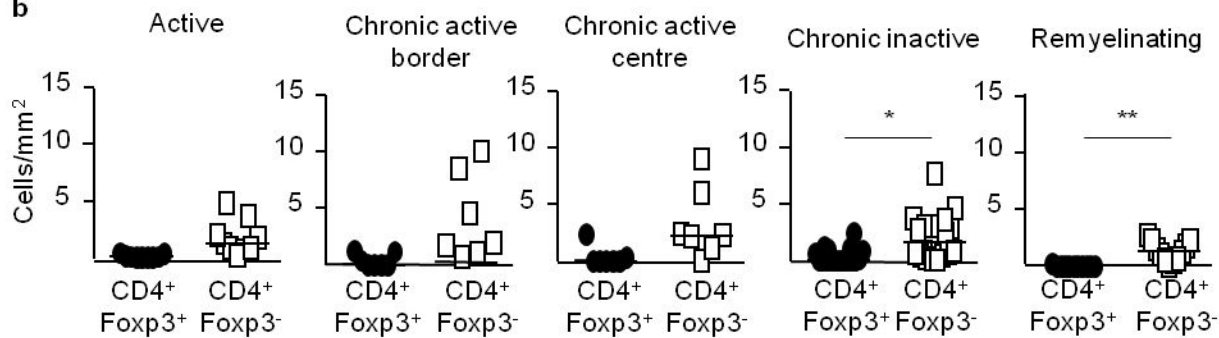
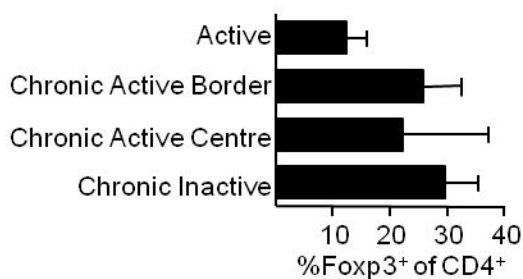
315 Relationships between the frequencies of CD4⁺Foxp3⁻ cells (a), or CD4⁺Foxp3⁺ cells (b), and the
316 frequencies of all cells staining for the indicated cytokine. Non-parametric 2-sided Spearman correlations
317 were used. Lesions were not segregated based on pathological type. 10 active lesions, 7 chronic active
318 lesions and 17 chronic inactive lesions were studied.

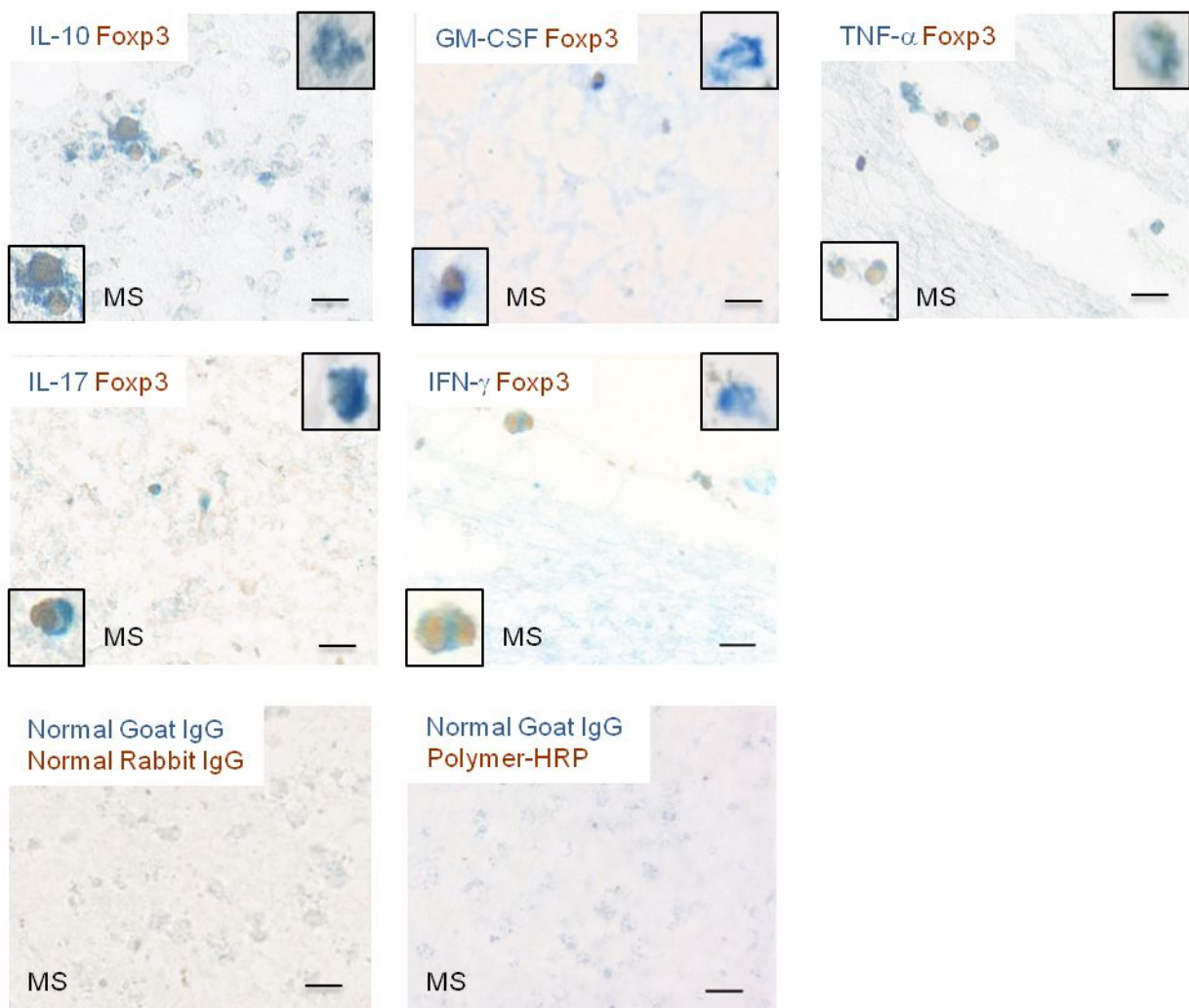
319

320 **Figure 4. Foxp3⁺ST2⁺ Treg are present in MS lesions.**

321 **(a)** Representative flow cytometry plots (gated on CD4⁺ cells) and summary data showing the expression
322 of ST2 in CD4⁺Foxp3⁺ cells in spleen, lymph nodes (LN) and CNS isolated from mice 16 days after
323 induction of EAE. A one-way ANOVA with Bonferroni's post test was used. **Graphs show means ±**
324 **SEM. 7 mice were studied.** **(b)** Representative immunohistochemistry image of IL-33 (brown) and
325 haematoxylin (blue) and summary data showing percentage of IL-33⁺ cells in the indicated human SPMS
326 lesions. **No staining was observed using isotype controls.** Scale bars 20 μm. **(c)** Representative
327 immunofluorescent staining for DAPI (blue), ST2 (green) and Foxp3 (red) in an active lesion. Arrows
328 delineate ST2⁺Foxp3⁺ cells (insets). **No staining was observed using isotype controls.** Scale bars 40 μm.
329 **(d)** Frequencies of Foxp3⁺ cells that stained for ST2 in the indicated SPMS lesions. 5 active lesions, 2
330 chronic active lesions and 6 chronic inactive lesions were studied. A Kruskal-Wallis test with Dunn's
331 multiple comparison correction was used. * p<0.05.

332

a**b****c**

a**b**