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

IL-10-producing, ST2-expressing Foxp3^{+} T cells in multiple sclerosis brain lesions

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

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Abstract

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

Main Text

$CD4^+Foxp3^+$ Treg cells control immune responses in inflamed tissues as well as secondary lymphoid organs[1, 2]. Treg cells isolated from the peripheral blood of MS patients are reported to show reduced suppressive function, but not reduced frequencies[3-5]. Treg cells can "trans-differentiate" to a pro-inflammatory function, producing IFN-γ or IL-17, when placed in conducive experimental conditions[6-8]. Peripheral blood Treg cells from MS patients were reported to display this ability, producing IFN-γ *in vitro* under the influence of IL-12[9]. The major drawback of such studies is that, out of necessity, only the peripheral blood of MS patients can be sampled and not the central nervous system (CNS) itself. Tissue inflammation can stabilize, rather than diminish Treg suppressive function. We reported that the accumulation of highly activated and suppressive, IL-10-producing Treg cells within the CNS is necessary for the natural resolution of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS[10, 11]. In addition, these CNS Treg cells resisted conversion to pro-inflammatory function *in vitro*[12]. Here, we sought to understand the distribution of Treg in human MS lesions and to gather evidence for suppressive, or pro-inflammatory roles for these cells.

Results and Discussion

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

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

Although the presence of CD4$^+$IL-17$^+$ T cells has been reported before[14], no analogous analysis has been made of cytokine production by Foxp3$^+$ cells in MS lesions. Two-colour immunohistochemistry identified co-expression of Foxp3 with IL-10, IL-17, IFN-γ, or GM-CSF in active and chronic lesions (Fig. 2a). Approximately 50% of Foxp3$^+$ cells stained positive for IL-10 (Fig. 2b). Lower frequencies of Foxp3$^+$ cells stained positive for pro-inflammatory cytokines. IL-10 was the dominant cytokine produced by Foxp3$^+$ cells (>60%) in active lesions and the borders of chronic active lesions (Fig. 2b). This was less evident in chronic inactive lesions and in the centres of chronic active lesions, where Foxp3$^+$ cells showed no enrichment in IL-10 over other cytokines. In contrast to IL-10, TNF-α staining in Foxp3$^+$ cells only became evident in chronic inactive lesions. Frequencies of Foxp3$^+$ cells staining for IFN-γ, IL-17, or GM-CSF were low in all active and chronic lesion types. We conclude that the predominant cytokine produced by Foxp3$^+$ cells within the brains of SPMS patients is IL-10. This is entirely consistent with our previous observations of Treg in the CNS of mice with EAE[10-12] and indicates that, in MS, Treg that infiltrate the lesions are in suppressive rather than pro-inflammatory mode.

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

Elegant murine studies have shown that IL-10 signalling in Treg cells is required for their own IL-10 expression and subsequent suppressive function[15]. Therefore it is plausible that, in addition to contributing to the IL-10 pool, IL-10$^+$ Treg cells are specifically attracted to, expanded in, or maintained

in lesions with high IL-10 levels. TNF-α-blockade is a potent therapeutic option for several human inflammatory diseases such as rheumatoid arthritis, Crohn's disease and psoriasis[16, 17], but not MS[18]. Studies on how TNF-α-blockade effects the Treg populations have led to conflicting results. TNF-α blockers have been reported to increase the number or function of Treg cells in RA and Crohn's[19, 20]. However, it has also been shown to inhibit suppressive function of Treg cells through down-regulation of Foxp3 in RA patients[21]. Recent studies indicate TNF-α signals selectively through TNFR2 in Treg cells[22, 23]. This suggests that Treg cells might require TNF-α for their suppressive function and provides a plausible explanation for the positive correlation between $Foxp3^+$ cells and $TNF\text{-}\alpha^+$ cells that we see.

Expression of the IL-33 receptor, ST2, has been associated with potent Treg function in murine models[24-26]. Indeed, we found ST2 to be particularly enriched in CNS Treg in EAE (Fig. 4a). IL-33 is highly expressed in the CNS in both EAE and MS (Fig. 4b)[27, 28]. Dual immunofluorescence identified the presence of $Foxp3^+ST2^+$ cells in MS brains (Fig. 4c). In particular, ~60% of $Foxp3^+$ cells in active lesions were $ST2^+$, whilst its expression was almost absent in $Foxp3^+$ cells in chronic lesions (Fig. 4d). High expression of both IL-10 (Fig. 2b) and ST2 (Fig. 4d) by $Foxp3^+$ cells in active lesions suggests that their suppressive potency should be greatest in these lesions and that this might wane in more chronic lesions. A recent study from Miron et al[29] demonstrated high numbers of M2 macrophages, also particularly in active lesions, of the same brain tissue studied here. This is interesting for two reasons. Firstly, IL-10 (perhaps originating from Treg cells) can promote the M2 phenotype, which is thought to contribute to remyelination by inducing oligodendrocyte differentiation. Secondly, a study of experimental cerebral malaria recently reported that IL-33 is protective by coordinating both Treg and M2 activity (the latter via expansion of type-2 innate lymphoid cells which release M2-promoting cytokines)[30]. Whether such a coordinated response is protective in CNS autoimmune inflammation, and whether there are viable therapeutic approaches that can boost the numbers and/or sustain the function of these cells, should be fruitful avenues for exploration.

109

Methods

Human Tissue specimens

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

Immunohistochemistry of T cell subsets

10 µM sections were fixed in 4% PFA (Fisher Scientific, Waltham, USA) and subsequently delipidised in 70% ice-cold ethanol. Antigens were retrieved using heating in acid citric buffer (Vector, Burlingame, USA). Sections were incubated with anti-Foxp3 (ab10563, rabbit, Abcam, Cambridge, UK) overnight at 4°C. Subsequently the sections were incubated with anti-CD4 (M7310, mouse, Dako, Glostrup, Denmark) for 30 minutes at room temperature. An EnVision G|2 Doublestain System, Rabbit/Mouse kit (Dako) was used for detection as per manufacturer's instructions, with exception of the use of an Vector Blue Alkaline Phosphatase Substrate Kit III (Vector) to develop the signal. Sections were mounted in aqueous permafluor medium (Thermo Scientific, Waltham, USA). Primary antibodies were omitted to check for non-specific binding of polymers. Rabbit IgG (ab27478, Abcam) or Mouse IgG1 isotype control (X0931, Dako) were used to control for non-specific binding of the primary antibodies. All IHC experiments were performed in triplicate.

Immunohistochemistry of cytokines

For double staining of Foxp3 and cytokines, combinations of antibodies against TNF-α, IFN-γ, IL-17, GM-CSF or IL-10 (AF-210-NA, AF-285-NA, AF-317-NA, AF-215-NA, AF-217-NA, all goat, R&D systems, Abingdon, UK) with anti-Foxp3 (rabbit, Abcam) were used. For single IL-33 staining a goat anti-IL-33 antibody (AF3625, R&D systems) was used. Briefly, frozen brain sections were fixed in 4% PFA (Fisher Scientific), followed by antigen retrieval as described above. Endogenous peroxidase was blocked with 3% H_2O_2 in dH_2O (Fisher Scientific), followed by blocking of biotin for 15 minutes (Vector). Sections were incubated with 10% horse serum in PBS (Biosera, Boussens, France) and Fc Receptor Blocking Solution was added (Human TruStain FcX Biolegend, London, UK). Primary antibodies were added overnight at 4°C. Cytokines were detected with donkey anti-goat-biotin (ab6578, Abcam) followed by streptavidin-alkaline phosphatase (SA-5100, Vector) and visualized with the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector). Slides were blocked with 10% goat serum in PBS (Biosera). Anti-Foxp3 (rabbit) was detected with an anti-rabbit polymer-HRP (Dako) and developed with DAB substrate (Dako). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, USA), and mounted in aqueous permafluor medium (Thermo Scientific). Secondary antibodies/polymers alone, or normal goat IgG (AB-108-C, R&D Systems) and rabbit IgG (Abcam) were used to control for non-specific binding.

Immunofluorescent staining of Foxp3 and ST2

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

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

EAE induction

C57BL/6 mice were bred under specific pathogen free conditions at the University of Edinburgh. All experiments were approved by the University of Edinburgh Ethical Review Committee and were performed in accordance with UK legislation. Female mice were used between 6-12 weeks old (n = 7). EAE was induced by administration 100µg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK, Cambridge Research Biochemicals, Teesside, UK), emulsified in complete Freund's adjuvant containing 200µg of heat-inactivated *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich), with a total volume of 100µl injected subcutaneously into the hind legs. On the same day and 48 hours later, 200ng of pertussis toxin (Health Protection Agency, Dorset, UK) was given in 0.5ml of PBS intraperitoneally. Clinical signs of EAE were assessed daily with the following scoring system: 0, no signs; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; 5, hindlimb paralysis with partial forelimb paralysis; 6, moribund or dead.

Isolation of CNS mononuclear cells and flow cytometry

Mice were sacrificed at d16 (when Treg were evident in the CNS) by CO_2 asphyxiation and perfused with PBS. Brains and spinal cords were removed, mechanically disrupted and digested in RPMI containing 7.5 mg/ml collagenase type 4 (Lorne Laboratories, Reading, UK) and 2.5 mg/ml DNAse I (Sigma-Aldrich) for 30 minutes at 37°C. Mononuclear cells were isolated from the interface of a 30%:70% discontinuous Percoll gradient (GE healthcare, Uppsala, Sweden) after centrifugation at 530xg for 20 minutes. Cells were stained using the following antibodies: anti-CD4 brilliant violet 650 (Biolegend), anti-Foxp3 eFluor 450 (eBioscience, San Diego, USA), anti-ST2 FITC (MdBioscience).

Data acquisition

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

Statistical analysis

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

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

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Figure legends

Figure 1. $CD4^+Foxp3^+$ T cell are enriched within MS lesions.

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

Figure 2. $Foxp3^+$ cells predominantly produce IL-10 in MS lesions.

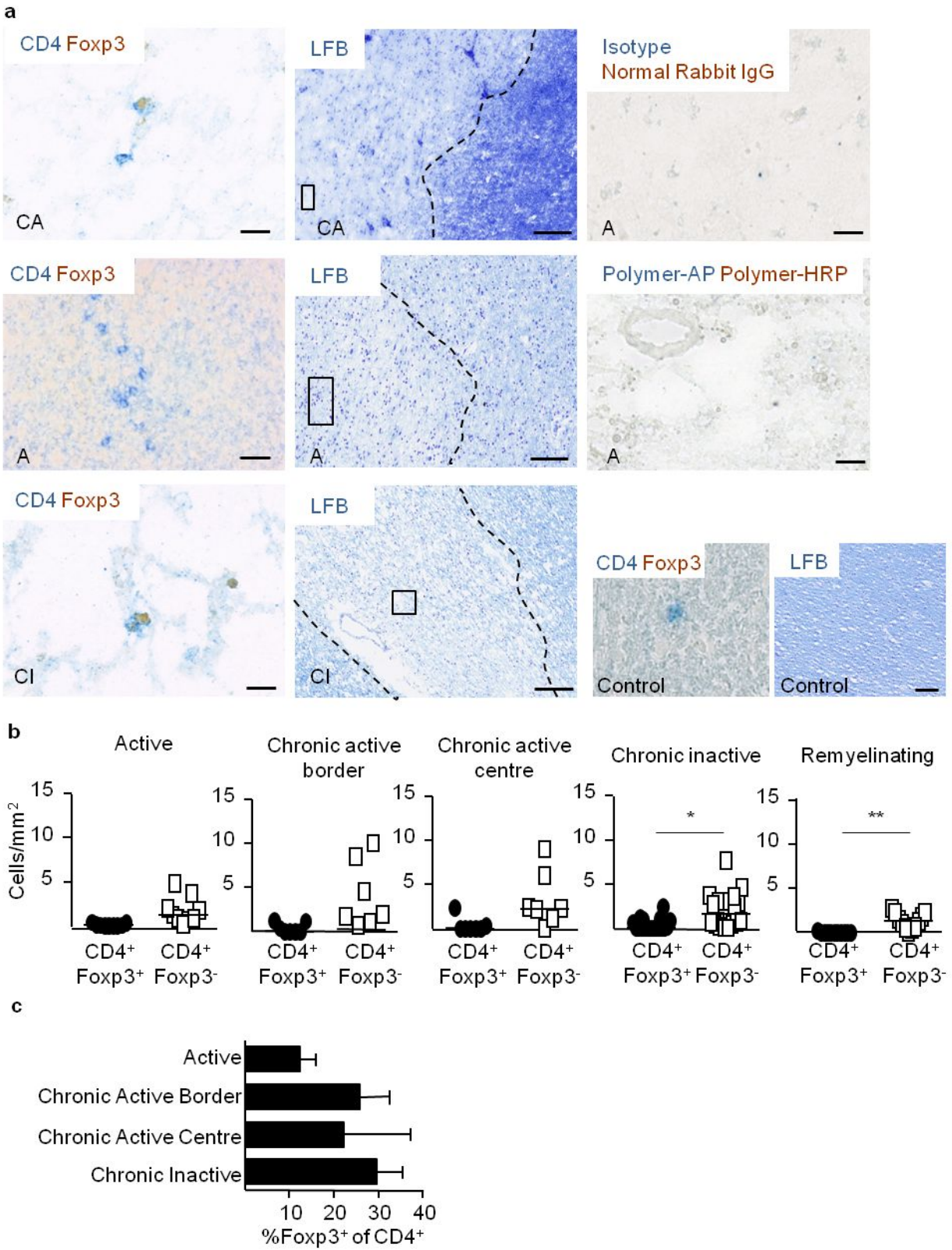
(a) Representative images of immunohistochemistry for individual cytokines (blue) and Foxp3 (brown). No staining was observed using isotype controls or secondary antibodies alone. Scale bars 20 µm. **(b)** Frequencies of $Foxp3^+$ cells co-staining for individual cytokines in the indicated SPMS lesion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

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

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

Figure 4. $Foxp3^+ST2^+$ Treg are present in MS lesions.

(a) Representative flow cytometry plots (gated on $CD4^+$ cells) and summary data showing the expression of ST2 in $CD4^+Foxp3^+$ cells in spleen, lymph nodes (LN) and CNS isolated from mice 16 days after induction of EAE. A one-way ANOVA with Bonferroni's post test was used. Graphs show means ± SEM. 7 mice were studied. **(b)** Representative immunohistochemistry image of IL-33 (brown) and haematoxylin (blue) and summary data showing percentage of $IL\text{-}33^+$ cells in the indicated human SPMS lesions. No staining was observed using isotype controls. Scale bars 20 µm. **(c)** Representative immunofluorescent staining for DAPI (blue), ST2 (green) and Foxp3 (red) in an active lesion. Arrows delineate $ST2^+Foxp3^+$ cells (insets). No staining was observed using isotype controls. Scale bars 40 µm. **(d)** Frequencies of $Foxp3^+$ cells that stained for ST2 in the indicated SPMS lesions. 5 active lesions, 2 chronic active lesions and 6 chronic inactive lesions were studied. A Kruskal-Wallis test with Dunn's multiple comparison correction was used. * $p<0.05$.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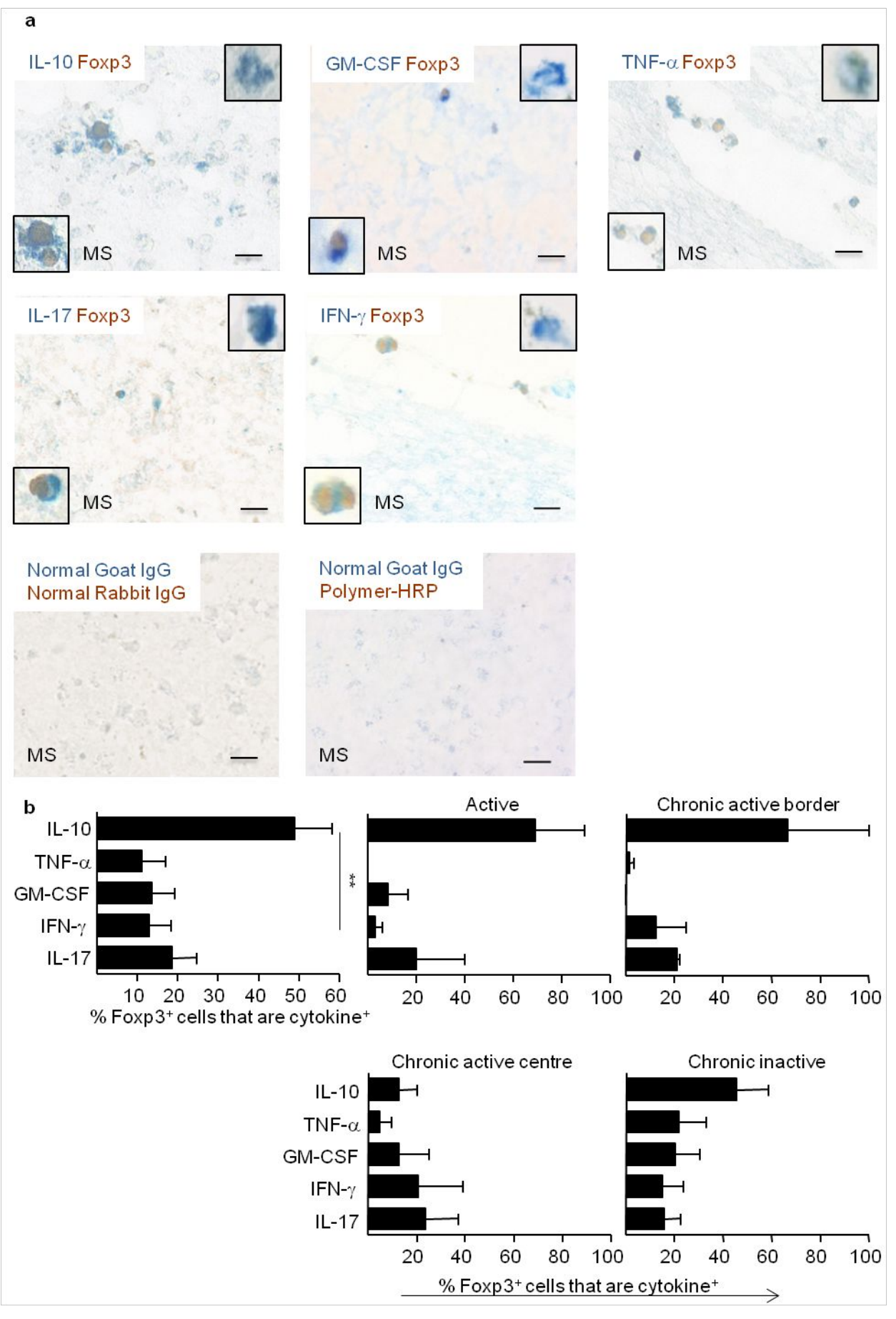
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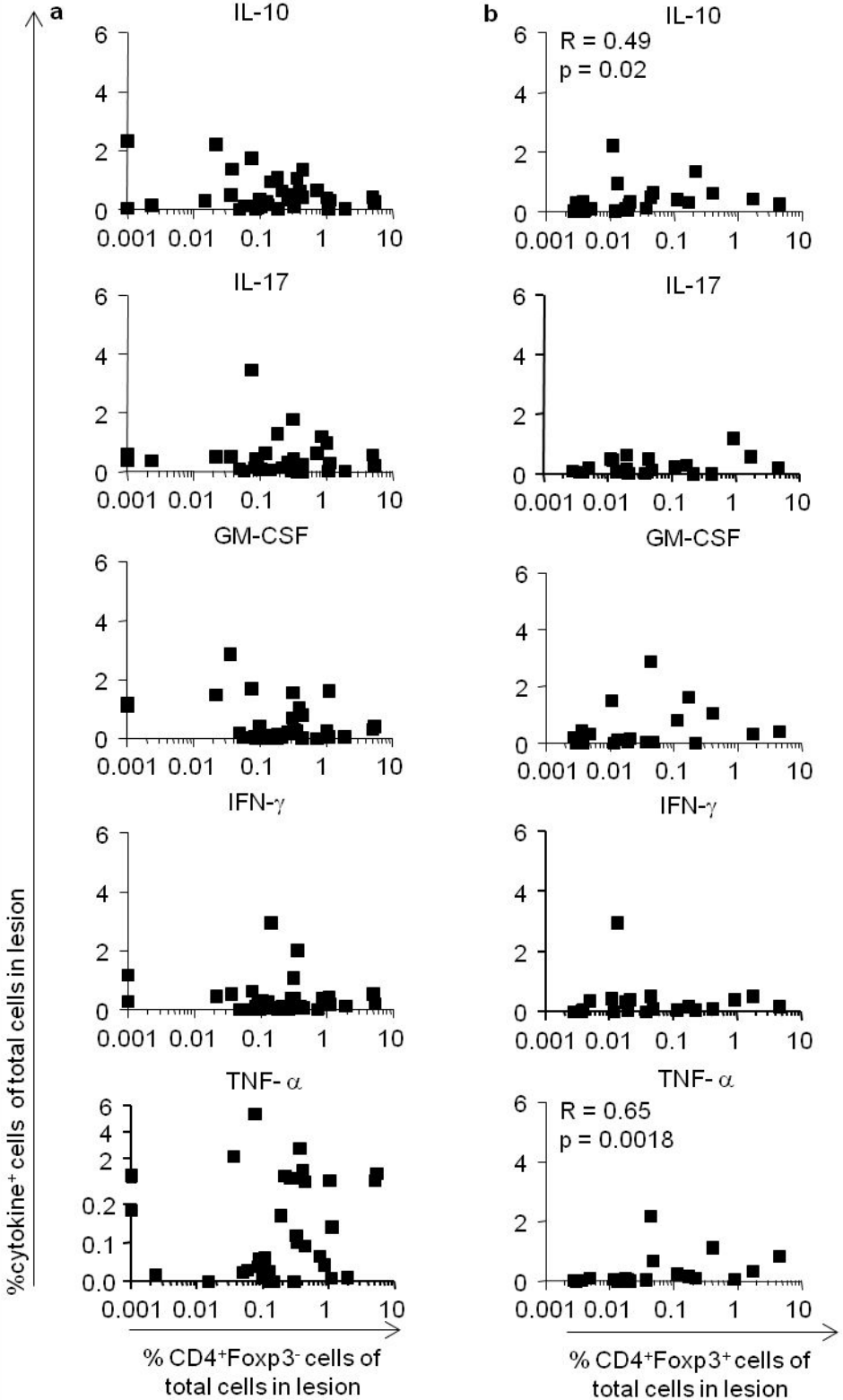
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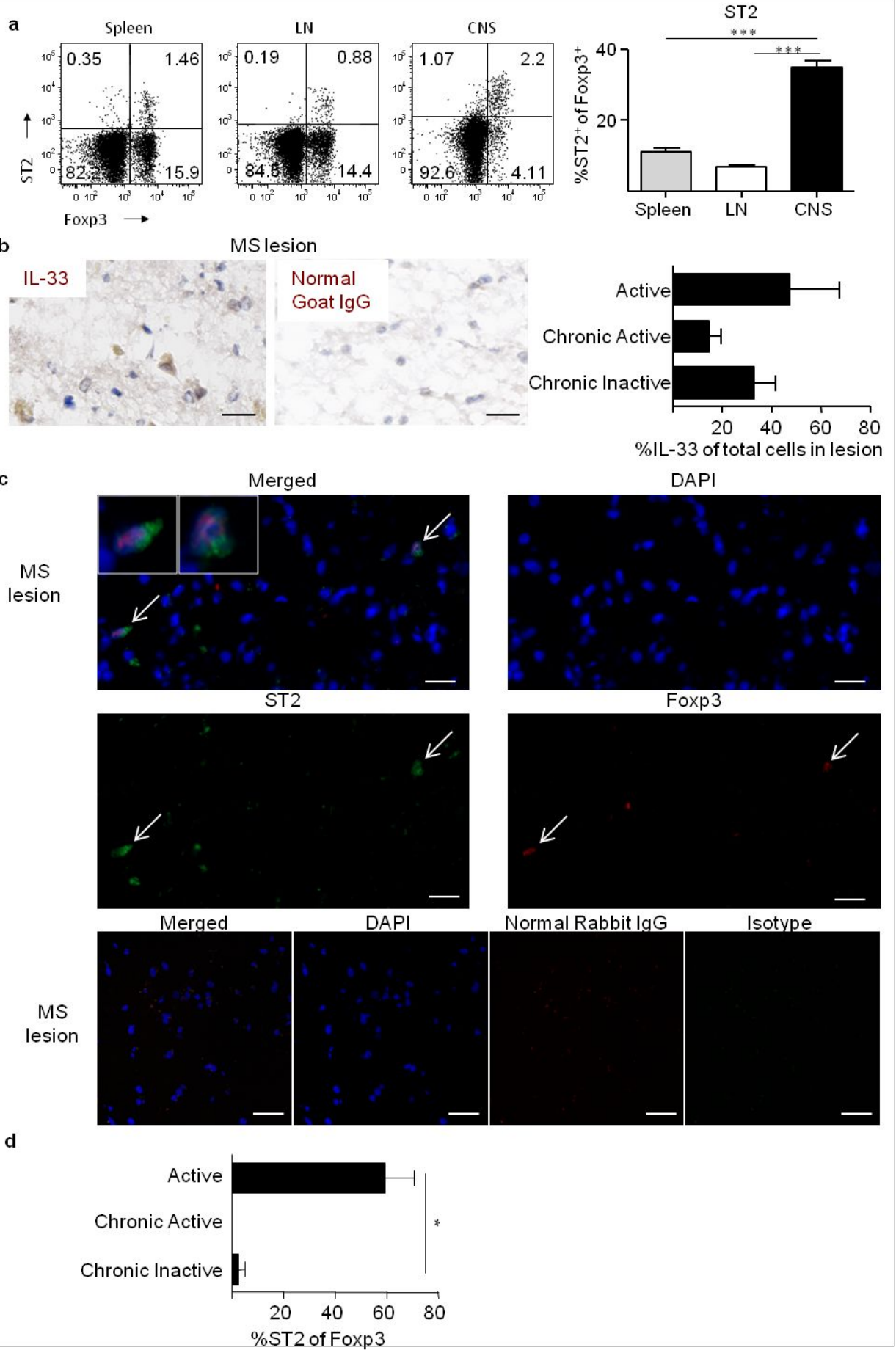
% CD4+Foxp3+ cells of total cells in lesion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Spleen
LN
CNS
0.35
1.46
82.1
15.9
0.19
0.88
84.8
14.4
1.07
2.2
92.6
4.11
ST2
Foxp3
ST2
***
***
%ST2+ of Foxp3+
40
20
Spleen
LN
CNS
b
MS lesion
IL-33
Normal Goat IgG
Active
Chronic Active
Chronic Inactive
20
40
60
80
%IL-33 of total cells in lesion
c
Merged
DAPI
MS lesion
ST2
Foxp3
Merged
DAPI
Normal Rabbit IgG
Isotype
MS lesion
d
Active
Chronic Active
Chronic Inactive
*
20
40
60
80
%ST2 of Foxp3