Glatiramer acetate reduces Th-17 inflammation and induces regulatory T-cells in the CNS of mice with relapsing–remitting or chronic EAE

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ABSTRACT

The aim of this study was to identify cell populations relevant to pathogenesis and repair within the injured CNS in mice that recovered from experimental autoimmune encephalomyelitis (EAE). We demonstrate that in two EAE models, with either relapsing–remitting or chronic course, T-cells and resident activated microglia manifested extensive IL-17 expression, with apparent localization within regions of myelin loss. In mice treated with glatiramer acetate (GA, Copaxone®), even when treatment started after disease exacerbation, CNS inflammation and Th-17 occurrence were drastically reduced, with parallel elevation in T-regulatory cells, indicating the immunomodulatory therapeutic consequences of GA treatment *in situ*.

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1. Introduction

In multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), the immune system provokes the pathological process via autoimmune inflammatory mechanisms (Hellings et al., 2002; Hohlfeld and Wekerle, 2004; Behi et al., 2005) leading to the characteristic demyelination and neurological damage (Bjartmar et al., 2003; Hobom et al., 2004). It is largely accepted that T-cells of the Th1 and Th2 subtypes are involved in the pathology and amelioration of these disease, respectively (Zamvil and Steinman, 1990). Recently, accumulating data indicate that Th-17 cells, which are induced through the transcription factors receptor-related orphan receptor (ROR)α and RORγt, produce the highly pro-inflammatory interleukin (IL)-17, and play an important role in the pathogenesis of inflammatory and autoimmune diseases (Bettelli et al., 2007; Aranami and Yamamaura, 2008). At the opposing pathway, the T-regulatory cells (Tregs), with their specific transcription factor forkhead box P3 (Foxp3), are potent immunosuppressors that ameliorate wide array of inflammatory conditions (Vila et al., 2009). Th-17 cells and Tregs have been demonstrated to arise from common precursors in a reciprocal manner, pending on the cytokine milieu, generating opposing outcomes, namely propagation or suppression of inflammation, respectively (Bettelli et al., 2006; Aranami and Yamamaura, 2008). Notably, recent findings support the notion that in the context of the inflammatory stimuli Tregs can convert to the Th17 phenotype,

resulting in excessive inflammation and tissue injury (Afzali et al., 2009; Zhou et al., 2009). These findings point to the importance of analyzing these cell populations in the target organ and following them in situ as a consequence of disease exacerbation and/or therapy.

Glatiramer acetate (GA, Copaxone®), an approved drug for MS treatment, is effective in the prevention and suppression of EAE induced by various encephalitogens in several species (Arnon and Sela, 2003). It has been shown that GA promotes neuroprotection and repair processes in the CNS, as manifested by the decrease in neurological damage (Gilgum-Sherki et al., 2003; Aharoni et al., 2005a) and demyelination (Aharoni et al., 2008), as well as by the increase in neurotrophic factors expression (Aharoni et al., 2005b), and neurogenesis (Aharoni et al., 2005a), in EAE mice treated by GA in comparison to untreated mice. The therapeutic activity of GA has been attributed to its immunomodulatory effect on various levels of the immune response. Thus, in the periphery GA was shown to bind promiscuously to major histocompatibility complex molecules (MHC), acting both as a MHC blocker (Fridkis-Hareli et al., 1994) and a T-cell receptor antagonist (Aharoni et al., 1999), leading to inhibition of various pathological effector functions. GA affected the properties of dendritic cells and monocytes, so that they preferentially stimulate Th2-like responses (Farina et al., 2005; Weber et al., 2007). Indeed, GA has been shown to be a potent inducer of Th2/3 cells that secrete high levels of anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor-β (TGF-β) (Aharoni et al., 1997). Furthermore, adoptively transferred GA-induced Th2/3 cells were detected in the CNS of recipient mice (Aharoni et al., 2000), expressing IL-10 and TGF-β, indicative of their in situ anti-inflammatory activity (Aharoni et al., 2003). The immunomodulatory effect of GA in the CNS was also

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evidenced by the corresponding decrease in IFN-γ, as well as by the bystander expression of Th2/3 cytokine in the resident astrocyte population. These cumulative results supported the notion that the effect of GA is mediated through immunomodulatory shift from the detrimental Th1 towards the anti-inflammatory Th2/3 response.

Recent studies indicated that the effect of GA on EAE/MS immunopathogenesis is not restricted to the Th2/3 versus Th1 pathways. Thus, it was shown that in vitro exposure of peripheral CD4+ T-cells, from healthy humans or from GA-immunized mice, to GA induced elevated levels of Tregs, through the activation of Foxp3 mRNA. Furthermore, in CD4+ T-cells of MS patients, whose Foxp3 level was low at baseline, GA treatment led to increased Foxp3 expression (Hong et al., 2005). Pretreatment of mice with GA, starting one week before the EAE induction resulted in increased expression of Foxp3 on Tregs during the mild disease which developed subsequently. Following their isolation from the spleen, these Tregs were more effective in EAE prevention than those isolated from untreated mice (Jee et al., 2007). Recently, using the chronic MOG-induced model, it has been demonstrated that GA treatment, starting one day after EAE induction, resulted in elevation of Foxp3 and reduction of Th-17 on the level of mRNA expression (Begum-Haque et al., 2008).

In the current study we attempted to identify cell populations that are relevant to pathogenesis and repair within the injured CNS. In particular we were interested in characterizing in situ the T-cell populations that are involved in therapeutic activity of GA when applied by the delayed (late) suppression regimen, namely after disease exacerbation had already occurred. To attend the issue of the multifaceted nature of MS, two EAE models with relapsing–remitting and chronic courses were utilized. We report herewith that in both EAE models, T-cells as well as activated microglia manifest extensive IL-17 expression in the CNS, with apparent localization within regions of myelin loss. In mice that recovered following GA treatment, CNS inflammation and Th-17 occurrence were drastically reduced with concomitant elevation in T-regulatory cells that had been generated in the periphery, but subsequent to disease exacerbation, accumulated in the inflamed CNS. The ability of the cells induced in the periphery by GA treatment to accumulate specifically in the injured CNS was demonstrated as well.

2. Materials and methods

2.1. Animals

C57BL/6 and (SJL/JxBALB/c)F1 mice were purchased from Harlan (Jerusalem, Israel). Female mice, 8–12 weeks of age, were kept under specific pathogen free (SPF) environment. All experiments were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute.

2.2. EAE

Relapsing–remitting EAE was induced in (SJL/JxBALB/c)F1 mice by the peptide encompassing amino acids 139–151 of proteolipid protein (PLP), synthesis by Novetide (Haifa Bay, Israel). Chronic EAE was induced in C57BL/6 mice by the peptide encompassing amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG), synthesis by Sigma (St. Louis, MO). Mice were injected subcutaneously at the flank, with 200 μl emulsion containing 200–300 μg of the encephalitogenic peptide in incomplete Freund's adjuvant enriched with 3 mg/ml heat-inactivated Mycobacterium tuberculosis (Sigma). Pertussis toxin (Sigma), 200–250 μg/mouse was injected intravenously immediately after the encephalitogenic injection and 48 h later. Mice were examined daily. EAE was scored as follows: 0—no disease, 1—limp tail, 2—hind limb paralysis, 3—paralysis of all four limbs, 4—moribund condition, and 5—death.

2.3. Glatiramer acetate (GA, Copaxone, Copolymer 1)

GA consists of acetate salts of synthetic polypeptides containing four amino acids L-alanine, L-glutamate, L-lysine, and L-tyrosine (Arnon and Sela, 2003). GA from batch 242902109, with an average molecular weight of 7400 kDa, obtained from Teva Pharmaceutical Industries (Petah Tiqva, Israel) was used throughout the study. GA treatment was applied by consecutive 7–8 daily subcutaneous injections (2 mg/mouse) either as early suppression treatment starting one day after disease induction (at day 1), or as late suppression treatment starting after the appearance of clinical manifestations (at days 18 and 13 in the PLP and the MOG experiments, respectively). A layout of the GA treatment schedules in characteristic experiments is demonstrated in Fig. 1A.

2.4. Perfusion and organ processing for immunohistochemistry

Animals were deeply anesthetized and perfused transcardially with 100 ml of Phosphate Buffered Saline (PBS). Brains were removed, postfixed in Bouin's solution (Sigma) for 12 h, followed by repeated washings with phosphate buffer (PBS). Organs were than cryoprotected with 15% sucrose solution and sectioned coronally (20 μm) by sliding microtome (Leica) through the entire brain. Free-floating sections were collected serially in PBS. Spinal cords were removed, snap frozen with isopentan (pre-cooled with liquid nitrogen) and sectioned sagitally (6 μm) by cryostat (Leica) with 50 μm skips.

2.5. Immunohistochemistry

Free-floating brain sections were pre-incubated in PBS solution containing 20% serum and 0.5% triton-x-100 for one hour, and then incubated overnight at room temperature with primary antibodies. Frozen spinal cord sections were treated with cooled acetone for 7 min at −20 °C, dried and incubated with 7% horse serum in antibody diluents (cell Marque) following 1 h incubation with the specific antibody. The following primary antibodies were used: rat anti-CD3 (Serotec) or rabbit anti-CD3 (NeoMarkeres, Fremont, CA), mouse anti-myelin basic protein (Abcam), rat anti-Mac-2 (Cedarlane,) goat anti-IL-17 (R&D Systems) and rat anti-Foxp3 (eBioscince). IL-17 staining was performed on Bouin's postfixed free-floating sections. Foxp3 staining was done mainly on frozen acetone treated sections. The second antibody step was performed by labeling with (donkey) highly cross-absorbed cy2, cy3 or Dylight-conjugated antibodies to rat, mouse, rabbit, or goat (Jackson ImmunoResearch, West Grove, PA), 1:200 for 20–40 min. Control slides were incubated with secondary antibody alone. In some cases in order to enhance the signal, we used biotinylated secondary antibodies for 90 min, followed by cy2 or cy3 conjugated Streptavidin (Jackson ImmunoResearch). Sections were stained with Hoechst 33258 (Molecular Probes) for nuclear counter staining.

2.6. Microscopy

Stained sections were examined and photographed by fluorescence microscope (E600, Nikon, Tokyo, Japan), equipped with Plan Fluor objectives connected to CCD camera (DMX1200F, Nikon), or by confocal microscope (Axiovert 100 M, Zeiss, Oberkochen, Germany). Digital images were collected and analyzed using image pro+ software. Images were assembled using Adobe Photoshop (Adobe Systems).

2.7. Lymphocyte isolation from spinal cords

Spinal cords were excised and the cervix portions were homogenized and dissociated to single-cell suspensions. The lymphocyte population (pooled from 2 mice for each treatment group) was isolated by separation on Percoll gradient (Amersham Pharmmacia), collecting the fraction between 30% and 60%.
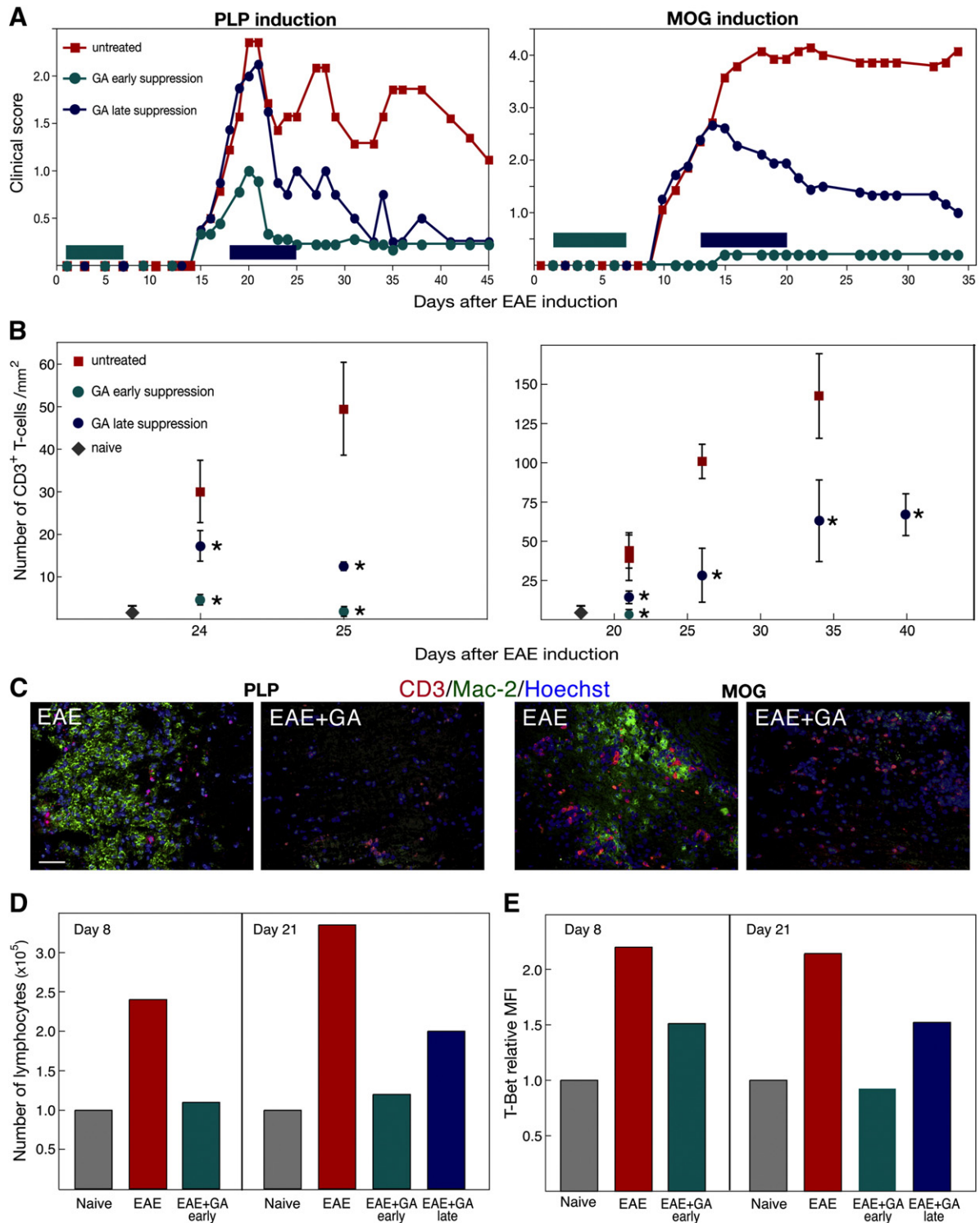
Fig. 1. The effect of GA on clinical and inflammatory manifestations of relapsing–remitting and chronic EAE. A. Clinical manifestations of EAE induced by PLP 139–151 peptide in (SJL/JxBALB/c)F1 mice (left) and MOG 35–55 peptide in C57BL/6 mice (right). GA treatment was applied by 7–8 daily injections starting either one day after disease induction (early suppression treatment), or after the appearance of clinical manifestations (late suppression treatment). The injection period of each treatment is illustrated along the *x* axis, 6–10 mice in each treatment group. The results depict averaged clinical score of one representative experiment from 5 to 6 performed for each model. B. Quantification of T-cells in spinal cord sections of PLP-induced (left) and MOG-induced (right) mice, stained immunohistochemically for CD3 expression. For each time point, CD3 positive cells were counted in 2–6 sagittal sections, with approximate size of 10 mm²/section, along the cervix. The average number of T-cells normalized per 1 mm² ± standard deviation is demonstrated. Asterisks indicate significant reduction compared to untreated EAE mice. C. Representative spinal cord (cervix) sections of PLP and MOG-induced mice 25 days after disease induction, stained for T-cells by anti-CD3 (red) and for activated microglia by anti-Mac-2 (green) antibodies and for overall nucleated cells by Hoechst (blue), depicting extensive inflammatory clusters in untreated mice and reduced inflammation in mice treated by GA - late suppression protocol. Scale bar indicates 50 μm. D. The number of lymphocytes isolated from spinal cord (cervix) of MOG-induced mice, 2 mice per treatment group, 8 and 21 days after disease induction. For each time point the number of cells from EAE or EAE + GA mice was normalized for the number of cells obtained by the naïve group from the same organ. E. FACS analysis of T-bet expression in CD4+ spinal cord isolated lymphocytes. The mean fluorescent intensity (MFI) relative to that of CD4+ lymphocytes from spinal cords of naïve mice is demonstrated. Results in D and E are from one representative experiment from two performed.

2.8. FACS staining and analysis

Cells were stained for surface markers by anti-CD4-Alexa 700 and anti-CD25-PE-Cy7 (eBioscience, San Diego, CA), at the recommended dilution for 30 min at 4 °C. For intracellular staining, cells were fixed and permeabilized with Foxp3 fixation/permeabilization buffer as recommended (eBioscience), then stained with anti-mouse Foxp3-APC, GATA3-FITC, RORγt-PE and T-bet-PerCP-Cy5.5 (eBioscience) for 30 min at 4 °C. Simultaneous negative-control staining reactions were performed with the appropriate isotype controls for each antibody. Stained cells were measured by BD LSR II Flow Cytometer instrument (BD Biosciences, San Jose, CA, USA) and FACS data were analyzed by Matlab (Mathworks, Natick, MA).

2.9. Cytokines secretion assay

Spleen lymphocytes (0.5×10^6/well) from naïve, EAE or EAE + GA mice were stimulated in vitro by medium alone, GA (50 μg/ml) or by immobilized anti-CD3 antibodies (5 μg/ml). Culture supernatants were collected after 3 days and tested for IL-17 and IL-5 concentration (pg/ml) by ELISA using the FlowCytomix Multiplex Kit (Th1/Th2 10plex; BenderMedSystems, Vienna, Austria).

2.10. Detection of GA-specific T-cells by in vivo imaging system (IVIS)

Spleen lymphocytes from mice that had been immunized with GA (2 mg/mouse, 10 daily subcutaneous injections) were activated in vitro by exposure to GA (50 μg/ml) and than labeled by the near-infrared lipophilic carbocyanine dye 1,1'-dioctadecyl-3.3.3'3'-tetra-methyliodotricabocyanine iodine (DiR, Invitrogen), for 45 min in 37 °C. The cells were than washed and injected (30×10^6 cells per mouse) into the peritoneum of mice inflicted with EAE (MOG or PLP -induced models), or with inflammatory bowel disease (dextran-induced model ref), or to control naïve mice. Cell injection was performed 10 days after EAE or IBD induction. Detection of the injected cells was performed using the whole body cooled CCD camera system (IVIS 100 series Imaging System, Xenogen, Alameda, CA).

2.11. Quantification and statistics

Quantitative analysis of Th-17 cells in the brain was performed by counting the double positive CD3+IL-17+ cells from the overall CD3+ T-cells, in 16 coronal sections, along each brain, starting at the level of the lateral orbital cortex with intervals of 0.55 mm (Bregma 2.3 to −6.5). Quantification of T-cells and T- regulatory cells in the spinal cord was performed by counting all the CD3+ cells and the double positive CD3+ Foxp3+ cells from the overall CD3+ population, in 2–6 sagittal sections with averaged size of 10 mm2 per section, along the cervix. Every counting was performed by scanning individual fields (0.05 in the brain and 0.13 mm2 in the spinal cord) using analysis software, along the entire section in 2–3 mice for treatment group for each time point. The results were subjected to one-way analysis of variance (ANOVA), followed by Fishers' LSD post-hoc comparisons where significant differences were found. The level of significance for all the tests was set at $p < 0.05$.

3. Results

3.1. The effect of GA on clinical and inflammatory manifestations of relapsing–remitting and chronic EAE

EAE manifestations as well as the effect of GA treatment were investigated in two MS models: the PLP 139–151 peptide-induced in (SJL/JxBALB/c)F1 mice, which is manifested in a relapsing–remitting course and the MOG 35–55 peptide-induced in C57BL/6 mice with a chronic disease course. GA treatment was applied to the EAE-induced mice by 7–8 daily injections starting either one day after disease induction (early suppression treatment), or after the appearance of clinical manifestations (late suppression, namely therapeutic treatment). A layout of these systems and the clinical scores observed in one characteristic experiment are demonstrated in Fig. 1A. GA treatment ameliorated the clinical manifestations in both EAE models when administered at either early or late staged of the disease: When treatment was initiated by early suppression, soon after disease induction, its effect was apparent, in the PLP model by development of a much less severe disease with only a single exacerbation, and in the MOG model, by complete prevention of disease development (only one from six mice showing grade 1 score). When GA treatment was applied after disease symptoms were apparent, it resulted in a substantial decline of the pre-existing clinical manifestations in both models. The beneficial effect of GA was sustained after treatment termination and lasted until the mice were sacrificed.

The in situ effect of GA treatment on CNS inflammation was manifested in both models in the number of T-cells counted in the spinal cord (in entire sagittal cervix sections) stained for CD3 expression in various time points after disease induction (Fig. 1B). In all the time points tested, GA treatment resulted in significant reduction in the numbers of CD3+ T-cells in comparison to EAE untreated mice. Furthermore, in the mice treated by the early suppression protocol CD3+ cells were rare similar to naïve mice. Representative pictures of spinal cord sections taken 25 days after EAE induction (Fig. 1C) depict the extensive widespread inflammatory clusters, containing T-cells (CD3+) and activated microglia (MAC-2+), in EAE mice of both models, with more pronounced T-cell involvement in the MOG-induced mice. In contrast, only a few small infiltrations were found in GA-treated mice even when treatment started after disease exacerbation, as described in the figure. In the mice treated before disease symptoms appearance (early suppression) inflammatory lesions were almost absent.

Correspondingly, the magnitude of the overall lymphocyte populations isolated from spinal cords (cervix) was elevated by 2.4 and 3.4 fold in MOG-induced mice compared with naïve controls, when evaluated either before or after disease appearance, namely 8 and 21 days after induction, respectively (Fig. 1D). In contrast, the number of spinal cord lymphocytes isolated from GA-treated mice was considerably smaller, 40% reduction from untreated mice by the late suppression regimen and restoration to the normal level by the early suppression treatment. Consistent with the CNS inflammation in EAE mice and the anti-inflammatory effect of GA, FACS analysis of spinal cord lymphocytes stained for the Th1 transcription factor T-bet revealed considerable elevation in the mean fluorescent intensity (MFI) of T-bet expression in EAE untreated mice, relatively to naïve controls, as well as its reduction by GA treatment (Fig. 1E). Treatment started after disease induction induced 30% decrease in T-bet expression from that of EAE untreated mice by day 8, and a total decline to the normal level by day 21. Even when treatment was started after disease appearance it led to a reduction by 30% of T-bet MFI in comparison to untreated mice, at the end of the treatment (in day 21). These cumulative results are indicative of the anti-inflammatory activity of GA on the CNS.

3.2. In situ IL-17 expression in relapsing–remitting and chronic EAE and the effect of GA on its expression

In view of the increasing evidence for the role of Th-17 in MS, it was of interest to investigate the involvement of the IL-17 effector pathway in the CNS pathology of EAE in both the relapsing–remitting and the chronic models, as well as in mice that had recovered from these diseases following GA treatment. Th-17 effector cells in the CNS were detected immunohistochemically by staining brain sections for IL-17 expression, and their T-cell identity was established by double staining for the T-subset marker CD3. As demonstrated in Fig. 2A,

brain section of EAE untreated mice, in both the PLP- and the MOG-induced models, contained multiple infiltrations of T-cells, and a considerable proportion of these cells manifested clear cytoplasmatic

staining with anti-IL-17 antibodies. In contrast, in brains of GA-treated mice we found smaller amounts of T-cells (in accord with the CD3 quantification in spinal cords demonstrated in Fig. 1B), and only a
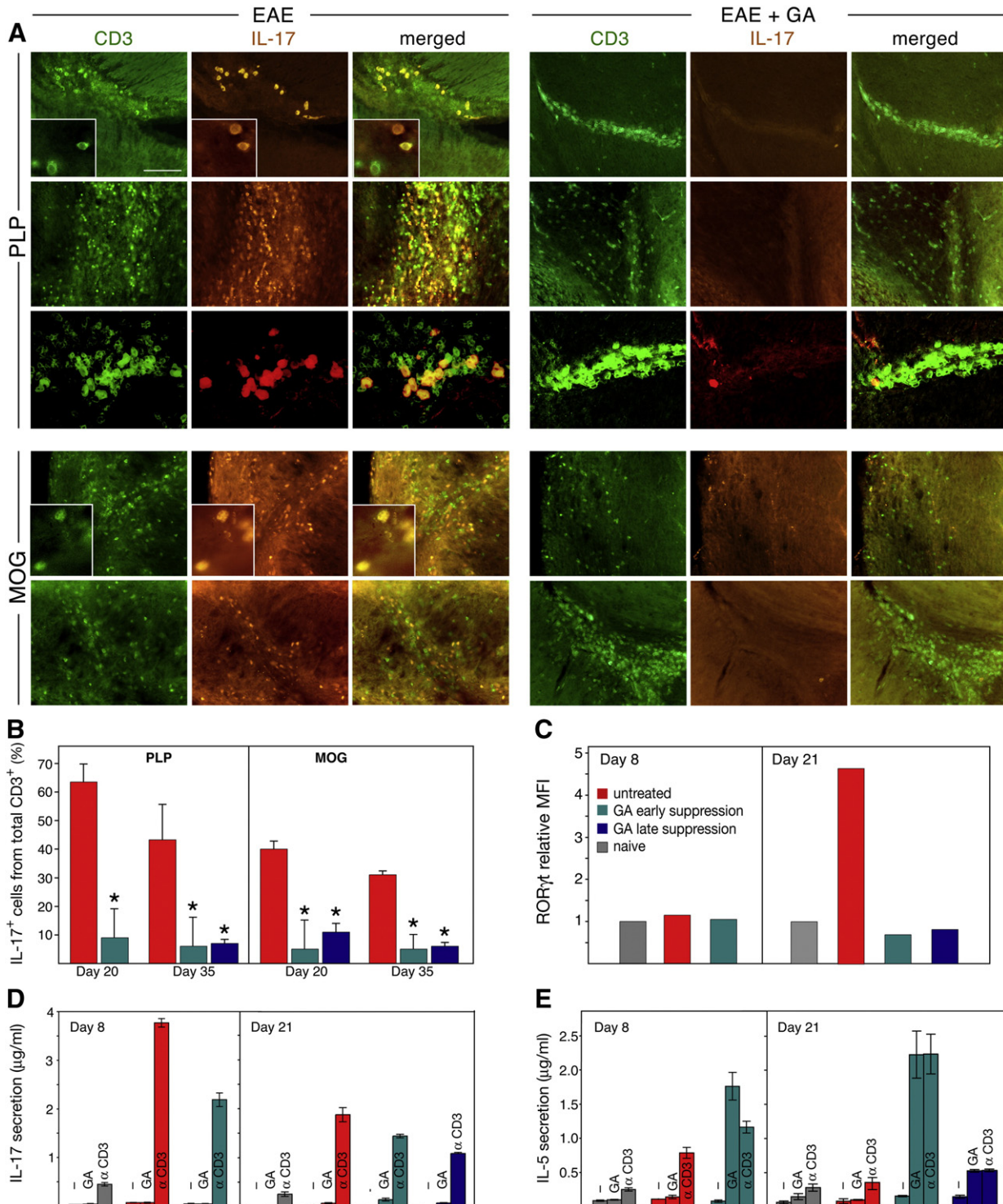

Fig. 2. IL-17 expression in PLP- and MOG- induced EAE and the effect of GA treatment. A. Immunohistological detection of Th-17 cells in brain sections of PLP and MOG-induced mice, stained for CD3 (green) and IL-17 (red). Representing images from white matter in the brain stem area depicting multiple double positive CD3 + IL-17+ cells in EAE mice, whereas in GA treated mice the majority of the CD3+ cells are negative for IL-17 expression. Scale bar indicates 100 μm for all rows except the third and the sixth of the PLP (confocal images) — 50 μm. B. Quantitative analysis of Th-17 cells in the PLP and MOG models, in brains of untreated and GA-treated mice, by early and late suppression. Each column represents the percentage of Th-17 expressing cells from the overall CD3+ T-cells, counted in 16 coronal sections, along each brain, 2–3 mice for each time point ± standard deviation. Asterisks indicate significant reduction from untreated EAE mice. C. FACS analysis of RORγt expression, gated on the CD4 marker, in lymphocytes isolated from spinal cords of MOG-induced mice, 2 mice per treatment group, 8 and 21 days after disease induction. MFI of RORγt expression relative to that of CD4+ lymphocytes from naïve mice is demonstrated. D. The effect of GA on the peripheral secretion of IL-17 (left) and IL-5 (right), in MOG-induced mice. Spleen lymphocytes were stimulated in vitro by GA (50 μg/ml) or by immobilized anti-CD3 antibodies (5 μg/ml), and their culture supernatants were tested by ELISA. The results in C and D are from one representative experiment from two performed.

small fraction of them were double positive for IL-17. Quantitative analysis of IL-17 expressing cells from the overall CD3+ T-cells (counted in 16 coronal sections, along each brain, in 2–3 mice for each time point, Fig. 2B) revealed that in untreated EAE mice, 20 days after disease induction, 64% and 40% of the T-cells co-expressed IL-17, in the PLP and the MOG models, respectively. This proportion somewhat declined by day 35, as 43% of the T-cells in the PLP and 31% in the MOG model expressed IL-17. GA administered by the early or late suppression regimen reduced the amounts of Th-17 cells in both models (significant differences from untreated mice for all the time points), below 11% in all treated mice.

To confirm the effect of GA on the abundance of TL-17 cells, lymphocytes isolated from spinal cords were stained for the transcription factor RORγt, which is involved in the differentiation of the Th-17 lineage. FACS analysis of spinal cord lymphocytes from MOG-induced mice, gated on the CD4 T-cell marker (Fig. 2C) revealed that before disease exacerbation (8 days after induction) there was no difference in RORγt expression in lymphocytes from EAE induced relatively to naive mice as manifested in the corresponding MFI values. In contrast, RORγt expression was elevated following disease development (at day 21) − MFI increased by 4.2 and 4.7 fold compared with naïve control in the two experiments performed. GA treatment applied before as well as after disease appearance eliminated RORγt elevation and reduced its MFI value to that found in naïve controls, thus indicating an inhibitory effect of GA on the IL-17 pathway.

Parallel to the effect of GA on IL-17 in the CNS, its effect in the periphery was followed by measuring IL-17 secretion in culture supernatants of spleen lymphocytes, that had been stimulated in vitro by GA or by anti-CD3 antibodies. The pattern of IL-17 secretion was compared to that of the prototype anti-inflammatory Th2 cytokine IL-5. As demonstrated in Fig. 2D, lymphocytes from GA-treated mice responded to GA stimulation by the secretion of high IL-5 concentrations (similar or higher than in response to anti-CD3), but not by the secretion of IL-17. Moreover, the amounts of IL-17 secreted in response to the overall stimulation by anti-CD3 antibodies were lower in lymphocytes cultures that had originated from GA-treated mice than in those obtained from untreated EAE mice. This is in contrast to their higher IL-5 secretion under similar conditions. These results indicate that GA treatment hinder IL-17 secretion in the periphery as well.

While counting the Th-17 cells in the brain, it was noticed that some IL-17 expressing cells did not carry the CD3 T-cell marker (demonstrated in Fig. 2A insert of the PLP model, which depicts two IL-17 expressing cells, of which only one expresses CD3). The IL-17+ CD3− cells were characterized by double staining with antibodies to the activated microglial marker MAC-2 and IL-17. As shown in Fig. 3, co-localization of corresponding fields on a single-cell level, revealed a fraction of double positive cells, indicating that IL-17 is expressed in the CNS not only by T-cells but also by the activated microglial population. The IL-17 positive microglia exhibited somewhat lower intensity of MAC-2 expression than their surrounding microglia cells, suggesting that they comprise a distinct subpopulation. It should be noted that Mac-2 is expressed not only by CNS resident microglia but also by monocytes and macrophages (Reichert and Rotshenker, 1999), the IL-17 expressing MAC-2 positive population in the CNS of EAE mice could represent hematogenous cells originated from the periphery. In contrast to the vast clusters found in the untreated EAE mice, only small accumulations of activated microglia cells were observed in EAE mice treated by GA, and no IL-17 expression could be identified in that population.

The relevance of IL-17 to EAE pathology was addressed also by looking into the localization of IL-17 expressing cells in the brain, in respect to demyelinated regions, by co-staining with anti-myelin basic protein (MBP) antibodies. Multiple myelin damaged sites were observed in various white matter regions of EAE inflicted mice (with clinical score 2–4) in both EAE models. This is demonstrated in Fig. 4
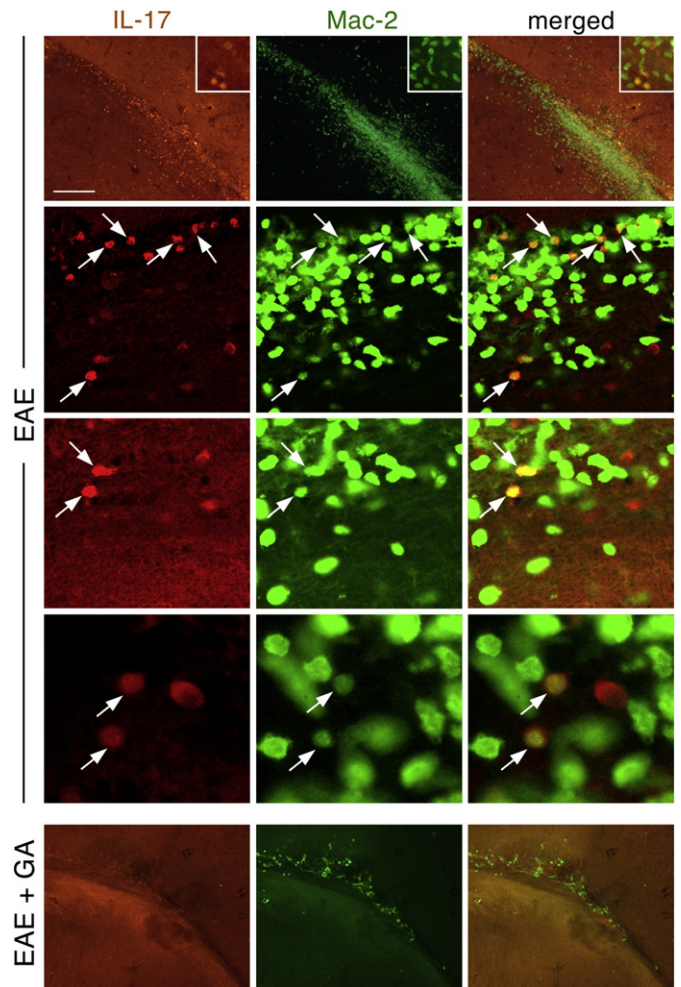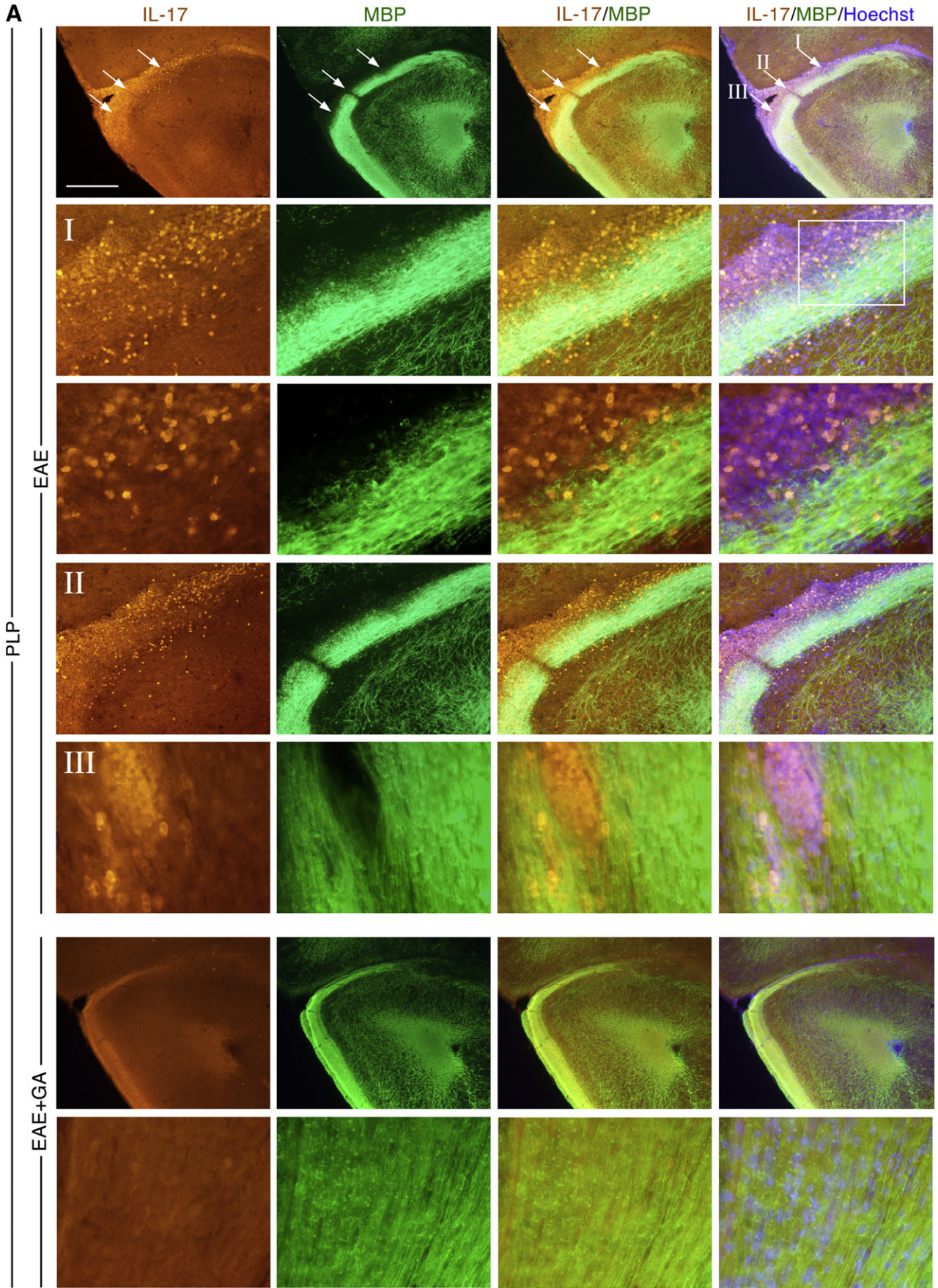

Fig. 3. IL-17 expression by activated microglia. Immunohistological detection of Th-17 cells in brain sections of PLP-induced mice 35 days after disease iduction, stained for IL-17 (red) and the activated microglial marker Mac-2 (green). Representing images from white matter area in the forebrain, depicting multiple double positive IL-17+ MAC-2+ cells. Scale bar indicates 200 μm, for the upper and lower rows, 100 μm and 25 μm for the second and third rows, respectively, (confocal images), and 20 μm for the fourth row.

by three adjacent injury sites in the lateral orbital cortex of a PLP-induced mouse, and by a widespread area of myelin loss in the inferior cerebral peduncle of a MOG-induced mouse. Myelin lesion sites were accompanied by cellular infiltrations (indicated by Hoechst labeling of overall nucleated cells), and in particular by extensive accumulation of IL-17 expressing cells. This observation is consistent with a detrimental role of IL-17 in EAE pathology. In brains of EAE mice treated by GA, both myelin damage and IL-17 expression were extremely lower, especially in the mice treated by the early suppression regimen, in which myelin loss was practically not detected.

3.3. The effect of GA on T-regulatory cells in the CNS of relapsing–remitting and chronic EAE mice

To further characterize the T-cell population relevant to the GA therapeutic activity, particularly the in situ involvement of regulatory cells, we investigated Tregs occurrence in the CNS of PLP and MOG-induced EAE mice, following the two GA treatment regimens. Tregs were detected by their distinctive transcription factor Foxp3 co-expressed either with the T-cell marker CD3
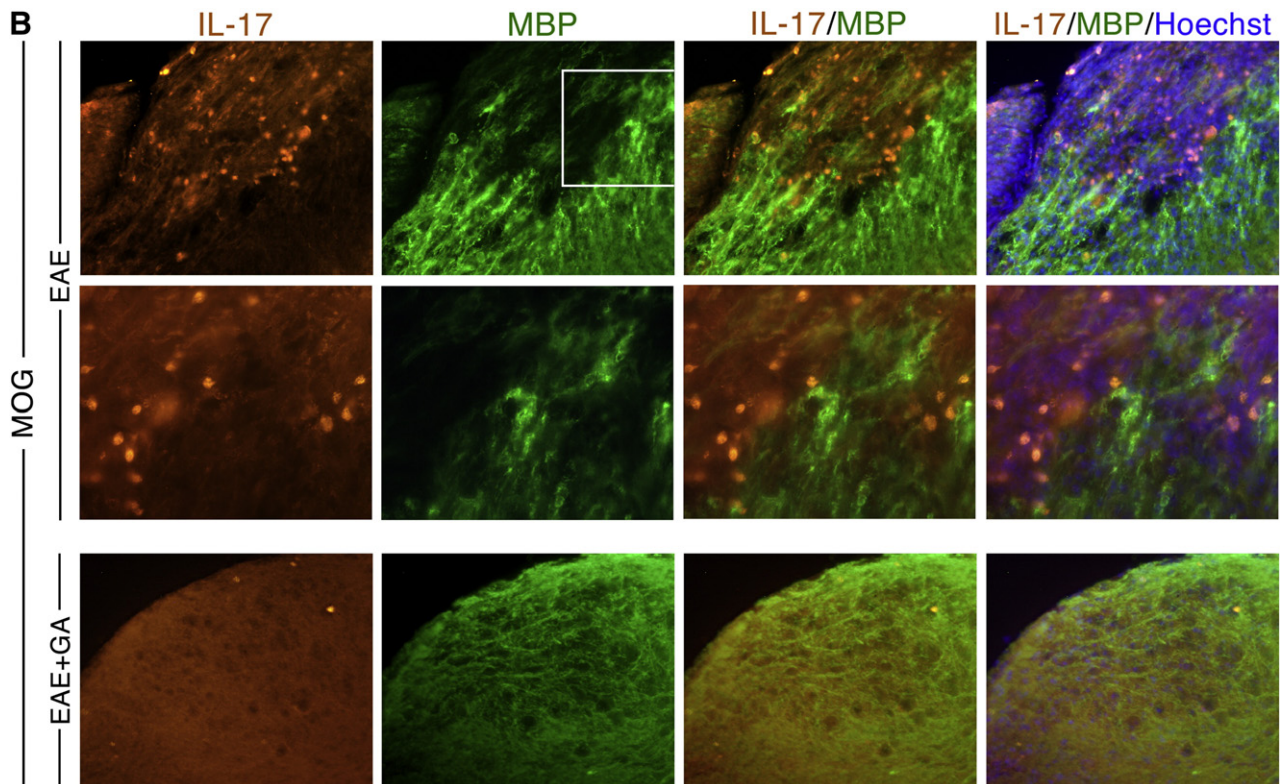
Fig. 4. Correlation of IL-17 expression with myelin damage. Staining of brain sections from EAE mice (day 35 after disease induction) with anti-IL-17 (red), anti-MBP (green) and Hoechst (overall nucleated cells, blue), indicating co-localization of IL-17 expressing cells in sites of myelin damage. A. Brain Sections from the lateral orbital cortex of a PLP-induced mouse. B. Brain Sections from the inferior cerebral peduncle of a MOG-induced mouse. Scale bar indicates 500 μm for the first and sixth rows in A, 200 μm for the fourth row in A, 100 μm for the second row in A, and first and third rows in B, and 50 μm for the third, fifth and seventh rows in A and second row in B.

applying immunohistochemistry, or with CD4 (gated on additional Treg marker — CD25) evaluated by FACS analysis.

Immunohistological analysis (shown in Fig. 5 for spinal cord sections) revealed only a few Foxp3 expressing cells in the multiple widespread CD3+ infiltrations in the CNS of untreated EAE mice of both PLP and MOG-induced models (A). Quantification of Foxp3 expressing cells from the overall CD3+ T-cell population (counted in 2–6 sagittal sections with averaged size of 10 mm2 along the cervix, for each time point, Fig. 5B) indicated gradual elevation of Tregs with time in untreated EAE mice, reaching maximum of 14% and 20% in the PLP and the MOG models, respectively. In contrast, in the GA-treated mice, although the total T-cell number was smaller than in untreated mice, higher proportion of double positive CD3+Foxp3+ was found, (significant elevation from untreated for all the time points tested). The Tregs fraction in the GA treated mice reached 31% from the total T-cell population in the PLP model (by day 25), and 37% in the MOG model (by day 40, in which the untreated mice already succumbed to the disease). Thus, GA treatment induced 2–3 fold elevation in Treg frequency, in comparison to untreated mice, in both models. It should be noted that using the immunohistological approach, elevation in Tregs was found only in GA mice treated by the late suppression protocol. Mice treated soon after disease induction, in which disease development had essentially been prevented, revealed only rare incidence of CD3 or CD3+Foxp3+ expressing cells, similar to their presence in naïve mice.

FACS analysis of lymphocytes isolated from spinal cords of MOG-induced EAE mice indicated, prior to disease exacerbation (8 days after induction), similar MFI values of Foxp3 expression in EAE-induced treated or untreated mice, relative to naïve controls (Fig. 5C). After disease development (at day 21), Foxp3 expression in untreated mice

was still only marginally elevated, while in spinal cord lymphocytes of GA treated mice there was considerable increase (2.3 and 2.6 fold compared with naïve, by the early and the late suppression treatments, respectively, similar results in two experiments). Thus, GA treatment applied after disease appearance induced 2–3 fold elevation in the expression of the Treg transcription factor Foxp3 by spinal cord CD4+ lymphocytes, consistent with the results obtained by the immunohistological analysis. Interestingly, parallel FACS analysis of Foxp3 expression in the peripheral (splenic) lymphocytes of the same mice revealed elevation in Foxp3 levels before disease appearance (at day 8), in both EAE and EAE+GA mice. However, after disease exacerbation (at day 21), Foxp3 MFI values declined to the normal level in both untreated and GA-treated mice. These findings imply that Tregs are generated in the periphery, but subsequently to disease exacerbation they accumulate in the inflamed CNS.

The overall consequence of GA treatment on both Th-17 and Tregs in brain sections of MOG-induced mice that were co-stained for IL-17 and Foxp3 is depicted in Fig. 6. Both markers exhibited distinct expression patterns, as evidenced by the absence of overlapping double positive cells. Notably, in brains of EAE untreated mice, cellular infiltrations (indicated by Hoechst labeling of all nucleated cells) contained numerous IL-17+ and minimal Foxp3+ cells, whereas the converse tendency of rare IL-17+ and many Foxp3+ cells was prominent in brains of GA treated mice.

3.4. Recruitment of GA-induced cells into the CNS of EAE affected mice

In an attempt to further understand the way by which GA injection generates therapeutic effects in situ, we investigated the ability of GA-induced lymphocytes, adoptively transferred into the periphery

of EAE inflicted mice, to accumulate specifically in the injured CNS along time. Lymphocytes from whole spleen population of mice that had been daily injected by GA, were stimulated by in vitro exposure to GA, and thereafter exogenously labeled by DiR and adoptively transferred into the peritoneum of mice inflicted by EAE. Naïve mice or mice suffering from an irrelevant autoimmune disorder – DSS-induced inflammatory bowel disease (IBD) – served as controls. Cells tracing in brain, spleen and intestine was performed at various time points by an in vivo imaging system (IVIS) on the level of different organs.

As shown in Fig. 7, GA-induced lymphocytes, injected to the periphery, localized in brains of EAE-induced mice (and not in brains of IBD or naïve mice), and their amount increased with time after cell transfer (Fig. 7). Whereas in EAE mice the GA-induced cells accumulated in the brain, in the gut their presence was marginal. In contrast, in IBD-induced mice most of the GA-specific cells accumulated in the intestines. Similar results were obtained in both the PLP and the MOG-induced models of EAE. These findings further corroborate the recruitment of GA-induced populations into the CNS of EAE mice, with specificity for pre-existing injury.

4. Discussion

Recent years gave rise to better understanding of the immunological mechanisms involved in MS/EAE pathology and therapy. Thus, in addition to the previously implicated Th1 versus Th2/3 paradigm, Th-17 and T-regulatory cells have been recognized as pivotal effectors of disease exacerbation and suppression, respectively. In the present study we investigated these two cell populations within the injured CNS in both PLP- and MOG-induced EAE that served as models for relapsing–remitting and chronic MS, respectively. We also followed their fate in consequent to therapy by GA treatment.

EAE development is associated with overall CNS inflammation (Traugott, 1989; Prendergast and Anderton, 2009). This was manifested in this study by the expanded lymphocyte populations isolated from spinal cord and their high expression of the Th1 transcription factor T-Bet, by the increased numbers of T-cells counted in CNS sections, as well as by the presence of extensive widespread inflammatory clusters containing T-cell and activated microglia. In both EAE models, these inflammatory manifestations were augmented with disease progression, yet in the CNS of MOG-induced mice the presence of CD3+ T-cell was more pronounced, whereas in PLP-induced mice the involvement of the resident innate immune system, depicted by Mac-2 expressing microglia, was prominent. Notably, in both EAE models IL-17 expressing T-cells constituted a significant fraction of the typical CNS inflammation. Furthermore, IL-17 in the CNS of EAE mice was expressed not only by T-cells but also by cells expressing MAC-2, which is considered a marker for activated proliferating microglia (Lalancette-Hebert et al., 2007) as well as for monocytes and macrophages (Reichert and Rotshenker, 1999). IL-17 has been reported as a T-cell-specific cytokine (Yao et al., 1995), but recently the potential of microglial cell line to produce IL-17 mRNA, in response to IL-23 or IL-1β stimulation, was shown in vitro (Kawanokuchi et al., 2008). In the present study, IL-17 protein expression by Mac-2 positive cells i.e. microglia monocytes and/or macrophages in the CNS of EAE-induced mice were demonstrated in situ, supporting their detrimental role in the context of the inflamed CNS. The significance of IL-17 expansion in the CNS was revealed by the extensive occupancy of IL-17 expressing cells in lesion sites with myelin loss, in both the chronic and the relapsing–remitting EAE models. These findings corroborate the pivotal role of IL-17 in EAE/MS pathology.

Whereas the total number of T-cells in the CNS was elevated along time after disease induction, the fraction of Th-17 cells within the T-cell population declined, suggesting the expansion of additional T-cell populations, such as regulatory T-cells. Indeed we found gradual

elevation in Foxp3 positive Tregs in the CNS of EAE mice, indicating that even in untreated mice, repair mechanisms are stimulated in response to the pathological process.

To analyze the role of Th-17 suppression and/or Tregs stimulation in the therapeutic activity induced by GA, we studied these cell populations in EAE mice in which the daily injection treatment was initiated after the disease was fully manifested, similarly to the practice in MS patients. In mice that recovered following the GA treatment, the overall CNS inflammation was decreased, consistent with the anti-inflammatory effect of GA that had been established in experimental animals and humans (Arnon and Sela, 2003). Accordingly, in the GA treated mice, lesion areas with myelin loss were smaller in size and rare, similarly to previous demonstrations (Gilgum-Sherki et al., 2003; Aharoni et al., 2008). The consequences of GA on IL-17 were even more robust, as in GA treated mice IL-17 expressing T-cells and microglia were hardly detected, and RORγt expression was essentially identical to that of naïve controls. This effect is in accord with the inability of lymphocytes stimulated by GA to secrete IL-17, as well as to the deficient IL-17 secretion in response to overall anti-CD3 stimulation, similarly to previous studies indicating the inability of GA to stimulate the secretion of inflammatory cytokine (Aharoni et al., 1997). The Prevention of IL-17 elevation in mice, in which EAE was blocked by GA treatment initiated one day after disease induction, was previously demonstrated on the mRNA level (Begum-Haque et al., 2008) and confirmed here for the early suppression regimen on the protein expression level as well. More significant, however, is the drastic reduction in the CNS expression of IL-17 and its specific transcription factor generated by the late suppression treatment, which implies that the inhibitory effect of GA on the IL-17 pathway is a primary effect involved in its genuine therapeutic activity.

Parallel to the suppressive effect on Th-17, GA treatment of EAE inflicted mice affected the T-regulatory pathway; 2–3 fold elevation in CD3+Foxp3+ Tregs frequency, for all the time points tested, in both EAE models. This effect could result from conversion of CD4+CD25− to CD4+CD25+ regulatory cells through Foxp3 activation that had been demonstrated by exposure to GA in vitro, as well as from the increased Foxp3 expression in splenocytes of GA-immunized mice (Hong et al., 2005). It was also demonstrated that type II monocytes induced by GA treatment directed the differentiation of Tregs (Weber et al., 2007). In accord with these previous results, in this study we found 2–3 fold elevation in Foxp3 expression in lymphocytes isolated from spinal cord of GA treated mice. It should be noted that the results presented here demonstrate elevation in Tregs in the CNS only in GA mice treated by the late suppression protocol. CNS of mice treated soon after disease induction, in which disease development had essentially been prevented, revealed neither inflammation nor tissue damage, and Tregs incidence in these mice was rare, similar to naïve mice. Likewise, GA treatment of naïve mice did not affect the CNS (not shown), suggesting that the inflammatory damage is a prerequisite for the repair processes and their further augmentation by immuno-modulatory treatment. In this context it is of interest that increased Tregs numbers was recently demonstrated in the peripheral blood of MS patients treated by GA (Haas et al., 2009), indicating the relevance to human therapy as well.

The cumulative effect of GA treatment in EAE inflicted mice was demonstrated by the rare IL-17 expressing cells and the prominent T-regulatory cells in brains of GA treated mice, as opposed to the numerous IL-17 and minimal T-regulatory cells in EAE untreated mice. Th-17 cells and Tregs were shown to originate from common precursors in a reciprocal manner (Bettelli et al., 2006). Namely, naïve CD4+ T-cells differentiated into Tregs if they were activated in the presence of transforming growth factor (TGF)-β, whereas they preferentially differentiated into Th-17 cells in the presence of TGF-β with inflammatory cytokines. The consequence of GA treatment may be then be interpreted in relation to its ability to increase TGF-β
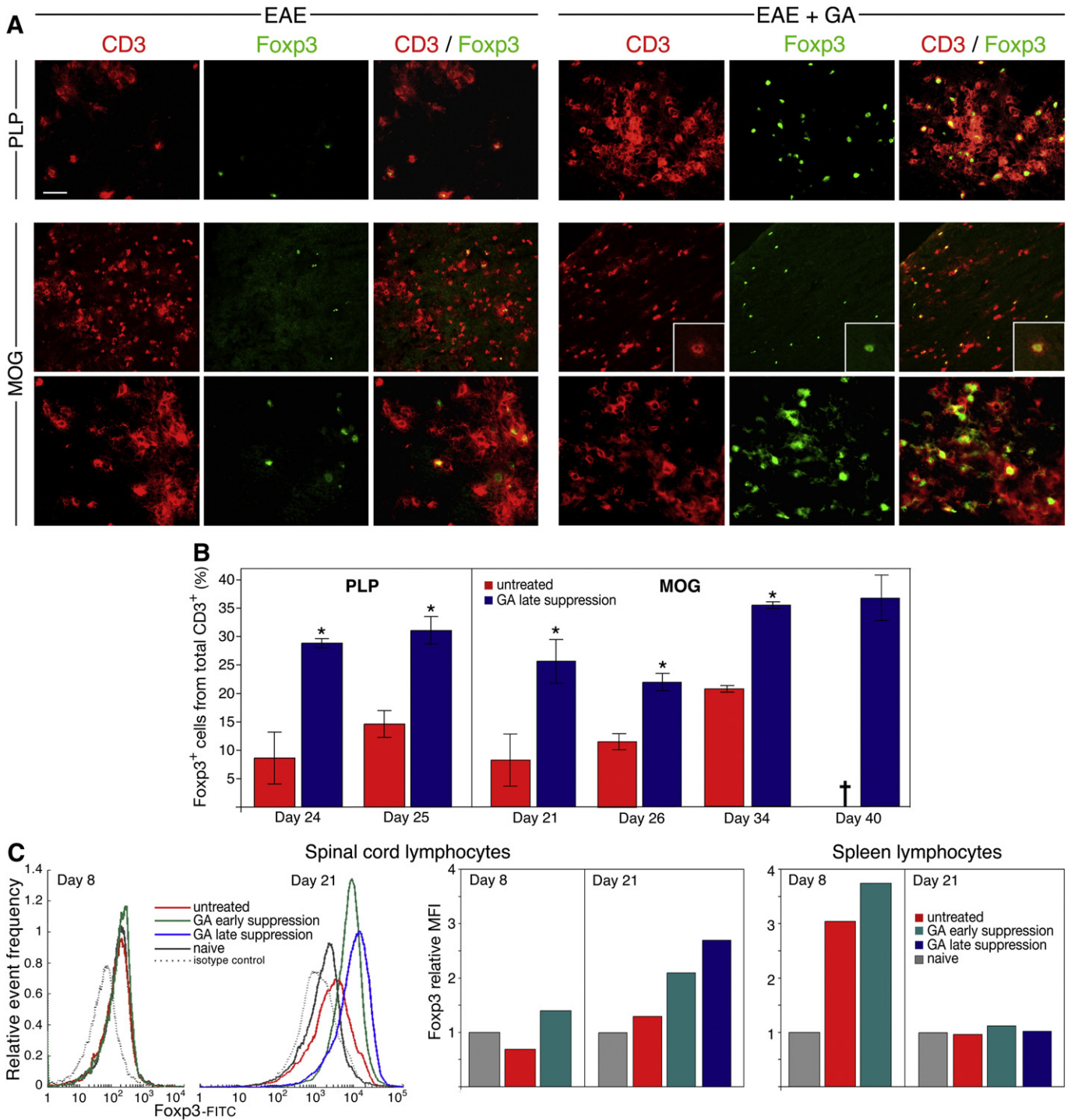
Fig. 5. The effect of GA on T-regulatory cells in the CNS of PLP- and MOG- induced mice. A. Immunohistological detection of Tregs in spinal cord sections of PLP (day 25) and MOG-induced mice (day 26), stained for CD3 (red) and Foxp3 (green). Representing images from white matter in the cervix depict a few CD3 + Foxp3+ cells in EAE untreated mice and their higher frequency in GA treated mice by the late suppression treatment. Scale bar indicates 50 μm in the first and third rows and 100 μm in the second row. B. Quantitative analysis of Tregs in the PLP and MOG models, in spinal cords of untreated mice and mice treated by GA after disease appearance. Each column represents the percentage of Foxp3 expressing cells from the overall CD3+ T-cells, counted in 2–6 sagittal sections with averaged size of 10 mm2 along the cervix, for each time point ± standard deviation. Asterisks indicate significant elevation compared with untreated EAE mice. † indicates that none of the mice in the untreated group survived. C. FACS analysis of Foxp3 expression. Left: Lymphocytes isolated from spinal cord. Measured histograms show level of Foxp3 in T-cells gated on CD4+ and CD25 high from the various treatment groups at days 8 and 21 after EAE induction. Bar plots present MFI values relative to naïve controls extracted from the histograms. Right: Relative values of CD4+ and CD25 high T-cells isolated from spleens of the same mice. 2 mice per treatment group, results from one representative experiment from two performed.

and decrease inflammatory cytokine levels, in the periphery as well as in the CNS (Aharoni et al., 2000, 2003), thus promoting the differentiation towards the regulatory pathway. This is especially important in view of the recent demonstration that in the context of inflammatory stimuli Tregs can subvert to the pathogenic Th17 phenotype, (Afzali et al., 2009; Zhou et al., 2009), thus emphasizing
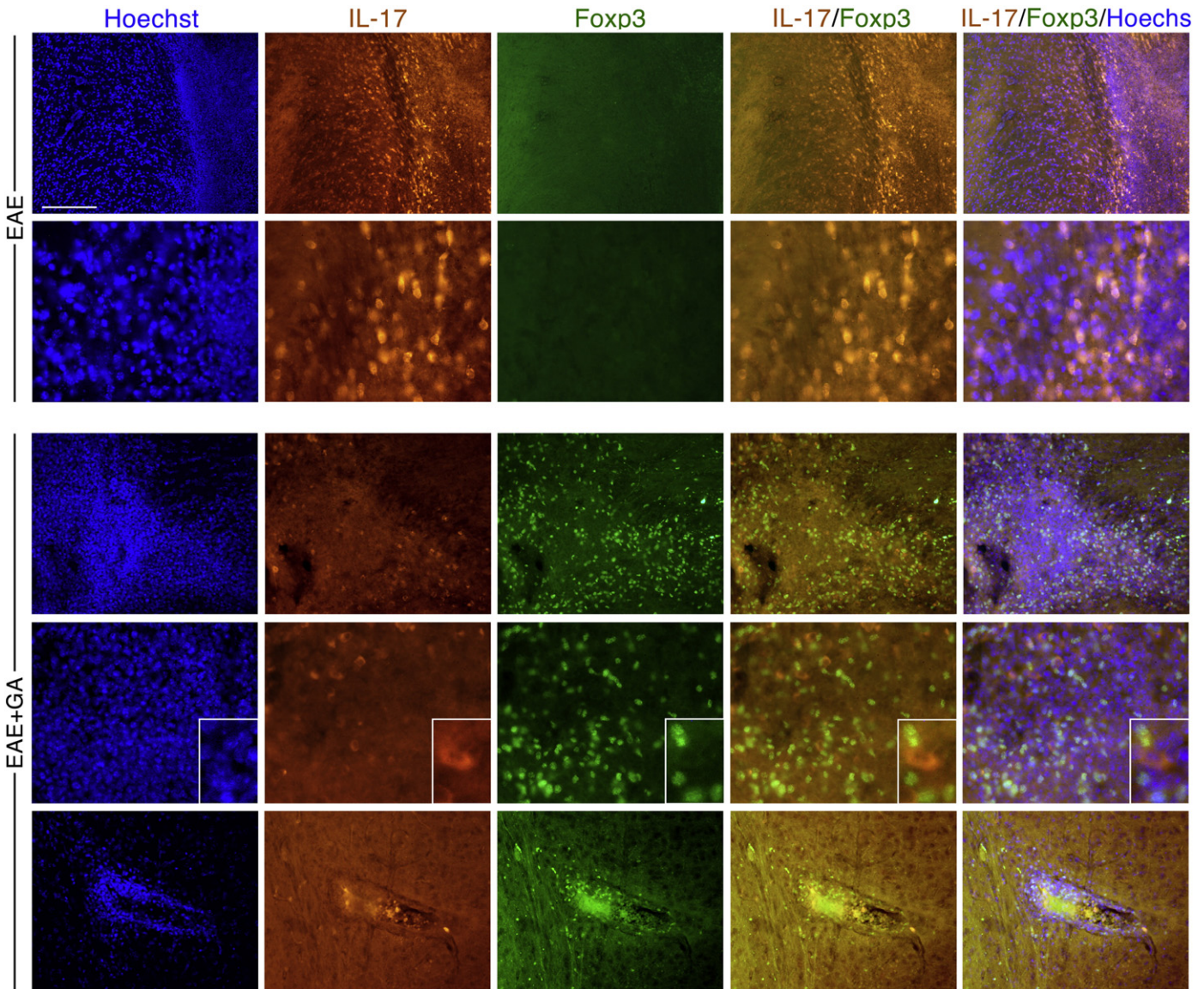
Fig. 6. The effect of GA treatment on Th-17 and Tregs in the CNS of EAE- induced mice. Staining of brain sections from MOG-induced mice with anti-IL-17 (red), anti-Foxp3 (green) and Hoechst (overall cells, blue), in the white matter area of the cortex and the thalamus (at the level of the dorsal hippocampus). In brains of EAE untreated mice cellular infiltrations contain numerous IL-17+ and minimal Foxp3+ cells, whereas in brains of GA treated mice, by the late suppression treatment, rare IL-17+ and many Foxp3+ cells are found. Note the perivascular cell infiltration in the lowest raw with predominantly Foxp3+ cells. Scale bar indicates 200 μm for the first row, 100 μm for the third and the fifth rows, and 50 μm for the second and forth rows.

the importance of maintaining immunomodulation in situ even when regulatory cells are recruited from external sources.

Tregs expansion occurred initially in the periphery, as demonstrated by the elevated Foxp3 expression in the spleen but not in spinal cord originating lymphocytes, before the appearance of clinical manifestations. Yet, after disease outbreak, Foxp3 level in spinal cord lymphocytes increased, in contrast to its decline in the spleen population. This finding implied that Tregs migrate through the interrupted blood brain barrier, into the inflamed CNS. The recruitment of cells generated in the periphery into the diseased organ was further confirmed by the restricted accumulation of GA-induced lymphocytes into the brain in EAE mice and into the intestines in IBD-induced mice, with specificity for pre-existing organ-specific injury. Thus, in EAE the expanded Tregs population, induced by GA injection in the periphery, can reach the inflamed CNS. In addition, GA induced Th2/3 cells from the periphery that have been shown to accumulate in

the CNS and to modulate the cytokines milieu (Aharoni et al., 2003), may support Tregs differentiation in situ.

The cumulative results of this study support the notion that IL-17 suppression with parallel elevation in T-regulatory cells are central mechanisms in the immunomodulatory effect of GA treatment in both relapsing–remitting and chronic models of MS. The GA induced T-regulatory cells alongside with the previously demonstrated Th2/3 cells are recruited into the CNS, enabling the immunomodulation that supports neuroprotective and repair therapeutic consequences.
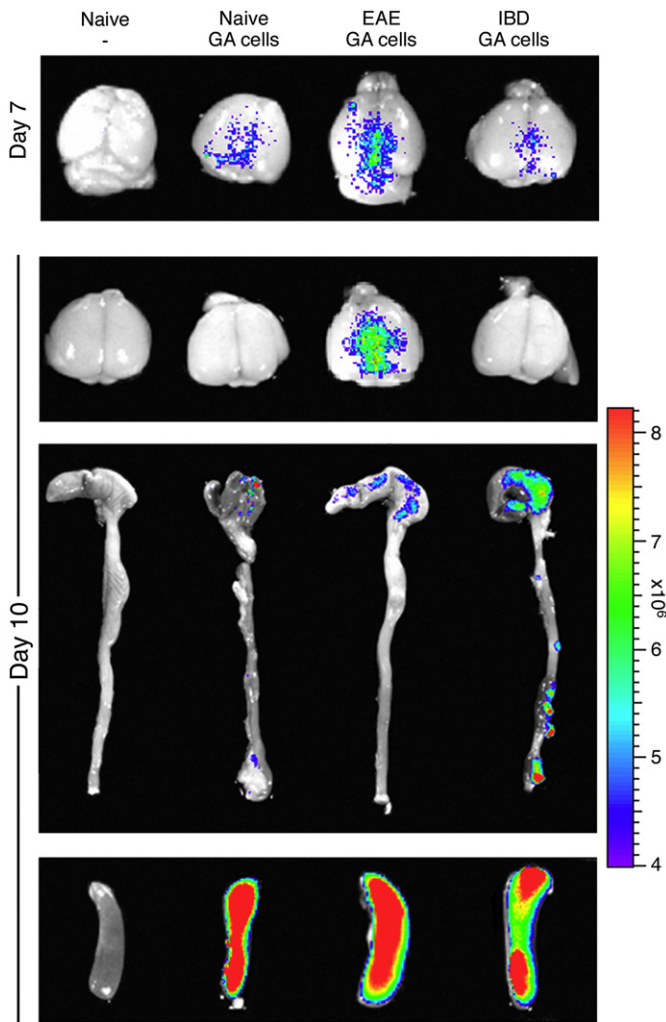
Acknowledgment

Fig. 7. Detection of GA-specific T-cells by in vivo imaging system (IVIS). TDIR-labeled GA-specific cells were adoptively transferred to mice inflicted with EAE (MOG-induced model), or with inflammatory bowel disease (dextran-induced model), 10 days after disease induction or to control naïve mice. IVIS imaging depicts brains from naïve, EAE and IBD mice, 7 days after cell transfer, as well as brains, intestine and spleens from naïve, EAE and IBD mice, 10 days after cells transfer. Pictures from one representative experiment from three performed.

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