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GCN2 kinase plays an important role triggering the remission phase of experimental autoimmune encephalomyelitis (EAE) in mice

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) has been widely employed as a model to study multiple sclerosis (MS) and indeed has allowed some important advances in our comprehension of MS pathogenesis. Several pieces of evidence suggest that infiltrating Th1 and Th17 lymphocytes are important players leading to CNS demyelination and lesion during the peak of murine EAE. Subsequently, effector T cell responses rapidly decline and the recovery phase of the disease strongly correlates with the expression of anti-inflammatory cytokines and the enrichment of Foxp3+ regulatory T (Treg) cells within the target organ. However, the mechanisms leading to the increased presence of Treg cells and to the remission phase of the disease are still poorly understood. Recent researches demonstrated that chemically induced amino-acid starvation response might suppress CNS immune activity. Here we verified an important participation of the general control nonrepressible 2 (GCN2), a key regulator kinase of the amino-acid starvation response, in the development of the remission phase of EAE in C57BL/6 mice. By immunizing wild type C57BL/6 (WT) and GCN2 knock-out mice (GCN2 KO) with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}), it was noticed that GCN2 KO mice did not develop the remission phase of the disease and this was associated with higher levels of CNS inflammation and increased presence of effector T cells (Th1/Th17). These animals also showed lower frequency of Treg cells within the CNS as compared to WT animals. Higher expression of indoleamine 2,3-dioxygenase (IDO) and higher frequency of plasmacytoid dendritic cells (pDCs) were found at the peak of the disease in the CNS of WT animals. Our results suggest that the GCN2 kinase-dependent sensing of IDO activity represents an important trigger to the EAE remission phase. The IDO-mediated immunoregulatory events may include the arresting of effector T cell responses and the differentiation/expansion of Treg cells within the target organ.

1. Introduction

For several decades, murine experimental autoimmune encephalomyelitis (EAE) has been studied as a model for immune-mediated central nervous system (CNS) demyelinating diseases, such as multiple sclerosis (MS) (Batoulis et al., 2011). Although many of the cellular and molecular mechanisms involved in the pathogenesis of these diseases are still not completely understood, pieces of evidence from *in vitro* and *in vivo* studies suggest that infiltrating Th1 and, most importantly, Th17 CD4+ lymphocytes are significant players leading to CNS inflammation and demyelination (Batoulis et al., 2011; Becher and Segal, 2011; Duong et al., 1994; Komiyama et al., 2006; Langrish et al., 2005; Merrill et al., 1992; Park et al., 2005). In EAE, Th17 cells are extensively found in CNS demyelinating lesions and, in some particular cases, adoptive transference of this CD4+ T cell subset to susceptible animals was shown to promote more severe symptoms than Th1 cells do (Langrish et al., 2005). Moreover, less severe symptoms of the disease are displayed by mice that lack the Th17 signature cytokine IL-17 (Komiyama et al., 2006) as well as by mice submitted to the neutralizing action of anti-IL-17 antibodies (Langrish et al., 2005).

In fact, it has been demonstrated that in C57BL/6 mice immunized with MOG_{35-55}, effector T cells start entering the CNS even before the development of the clinical EAE symptoms and the frequency of Th1 and Th17 cells in the target organ reaches the maximum around the peak of the disease (Korn et al., 2007; Murphy et al., 2010). The peak is also characterized by intense activation of microglia (Murphy et al., 2010) and recruitment of blood-derived CD11b+ myeloid cells in a CCR2-dependent manner (Ajami et al., 2011; Izikson et al., 2002). The peak is usually followed by a remission phase in which the animals, although not displaying

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complete recovery, do present significant improvements in the clinical signs. This remission phase is characterized by a decline in effector Th1 and Th17 cells within the CNS and an enrichment of Foxp3+ regulatory T (Treg) cells, which become much more frequent in the target organ at this stage of the disease (Korn et al., 2007). Rapid proliferation of Treg cells within the CNS has been demonstrated at this time point during disease. This Treg cell proliferation/expansion seems to drive a switch in the balance favoring Treg activity over effector T cell responses and correlates with recovery (O'Connor et al., 2007). Depletion of Treg cells has been shown to inhibit the natural recovery from EAE, demonstrating that the enrichment of Treg cells within the CNS during the course of EAE is essential for the occurrence of the remission phase (McGeachy et al., 2005).

Although these observations may explain the kinetics of EAE, describing an inflammatory profile at the peak of the disease followed by a regulatory stage that correlates with partial remission of the symptoms, the mechanisms triggering this shift are still not completely understood. Moreover, recent researches demonstrated that chemically induced GCN2-dependent amino-acid starvation response might suppress CNS immune activity by dampening Th17 cell responses (Sundrud et al., 2009).

Herein, we demonstrate that the GCN2 (general control nonrepressible 2) kinase, which is involved in the cellular amino acid starvation response (AAR) (de Haro et al., 1996; Dever and Hinnebusch, 2005), plays an important role as a trigger for the regulatory immune response that allows the development of the EAE remission phase.

2. Materials and methods

2.1. Mice

Foxp3^{GFP} knockin mice (background C57BL/6) were described elsewhere (Bettelli et al., 2006) and were kindly provided by Dr. Howard L. Weiner, Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School. GCN2-KO mice (background C57BL/6) (Roffé et al., 2013) were kindly provided by Prof. Beatriz Castilho, Federal University of Sao Paulo. Foxp3^{GFP} mice were crossed with GCN2-KO mice to obtain homozygous GCN2-KO Foxp3^{GFP} mice. Mice of 6–8 weeks old were used and maintained under specific pathogen-free conditions in the animal facilities at the Federal University of São Paulo. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experimentation at the Federal University of Sao Paulo.

2.2. Induction of EAE

EAE was induced by subcutaneous immunization with 200 μL of an emulsion containing 200 μg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; Proteimax, Brazil) and 400 μg of *Mycobacterium tuberculosis* extract H37 Ra (Difco) in incomplete Freund's adjuvant oil. In addition, the animals received 200 ng of pertussis toxin (Sigma–Aldrich) i.p. on day 0 and day 2. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tone in the tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, tetraplegia; 5, moribund.

2.3. Histopathology

Spinal cord tissue samples from MOG-immunized mice at the peak (day 15) and remission (day 21) phases of EAE were collected, fixed in 4% formaldehyde and paraffin-embedded. Sections of lumbar region were stained with hematoxylin and eosin (HE) or Luxol Fast Blue to assess inflammatory infiltration and demyelination, respectively.

2.4. Preparation of CNS mononuclear cells

Mice were perfused through the heart with cold PBS. Brain was dissected and spinal cords flushed out by hydrostatic pressure with PBS. Central nervous system tissue was cut into pieces and digested using Neural Tissue kit (P) (Miltenyi Biotec MACS). Mononuclear cells were isolated by passing the tissue through a cell strainer (70 μm), followed by a percoll (Sigma–Aldrich) gradient (37%/70%) centrifugation. Mononuclear cells were removed from the interphase, washed and suspended in PBS for FACS analysis.

2.5. Preparation of plasmacytoid dendritic cells (pDCs) and naïve T cell

Total splenic cells of Foxp3^{GFP} or GCN2-KO-Foxp3^{GFP} mice were FACS stained and submitted to cell sorting. CD4+CD62L+GPF− subset was sorted as naïve T cells. pDCs were sorted as CD317+CD3− from C57BL/6 WT mice. Purity of sorted subsets was typically >98% for naive T cells and >80% for pDCs. Cytometry antibodies were purchased from eBioscience. All samples were sorted in the FACSAria™ II (BD Biosciences).

2.6. Culture medium for in vitro assays

In vitro assays were performed in RPMI1640 medium supplemented with 10% FBS, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin.

2.7. Plasmacytoid dendritic cells (pDCs) and naïve T cells co-culture

pDCs and naïve T cells were co-cultured as described (Matteoli et al., 2010). Briefly, 2×10^4 sorted pDCs were co-cultured with 1×10^5 WT or GCN2-KO naïve T cells in the presence of 1 μg/mL soluble anti-mouse CD3e (eBioscience). Cells were cultured for 6 days. To promote Treg cell differentiation, 3 ng/mL of recombinant TGF-β1 (R&D Systems) was added in the 4th day of culture. Cells were then recovered and stained for FACS analysis.

2.8. In vitro differentiation of naïve T cells

Naïve T cells (3×10^5/well) WT or GCN2-KO were cultured for 5 days in presence of 1 μg/mL of plate-bound anti-mouse CD3e and 2 μg/mL of soluble anti-CD28. For Th17 differentiation, the following was added to the culture: IL-1β (10 ng/mL), IL-6 (40 ng/mL), IL-23 (10 ng/mL), TGF-β1 (2 ng/mL), TNFα (10 ng/mL), anti-mouse IFN-γ (10 μg/mL) and anti-mouse IL-4 (5 μg/mL); anti-mouse IL-2 (5 μg/mL) was added in the 3rd day. Th1 were differentiated in the presence of IL-12 (40 ng/mL), IL-2 (20 ng/mL) and anti-mouse IL-4 (5 μg/mL). Treg cells were generated by adding TGF-β1 (2 ng/mL). All recombinant cytokines were purchased from R&D Systems and neutralizing antibodies from eBioscience.

2.9. Intracellular cytokine staining

For intracellular staining of cytokines, SNC mononuclear cells or *in vitro* differentiated T cells were stimulated with PMA (50 ng/mL, Sigma–Aldrich), Ionomycin (1 mM, Sigma–Aldrich) and Brefeldin A (eBioscience) for 4 h. After staining of surface CD4, cells were fixed and made permeable using Cytofix/Cytoperm and Perm/Wash buffer from eBioscience according to the manufacturer's instructions. All antibodies to cytokines (IFN-γ, IL-17) were obtained from

eBiosciences. Samples were acquired on a FACSCanto™ II (BD Biosciences) and were analyzed by the software FlowJo, 9.3.3 version (Tree Star, Inc.).

2.10. RNA isolation and quantitative RT-PCR

Total RNA of spinal cord and sorted CNS infiltrating T CD3+CD4+ cells was obtained by Trizol® (Invitrogen) method following the manufacturer's instructions. cDNA was synthesized with SuperScript II reverse transcriptase kit (Invitrogen). Gene expression was evaluated by quantitative real-time PCR (qPCR) using the SybrGreen-based system of detection (Applied Biosystems) and the GAPDH gene as the reference for normalizations. Relative expression was calculated by ΔΔCt method (Schmittgen and Livak, 2008). We used 3 animals per group in each experiment. Reactions were performed in triplicates and results are representative of at least 2 independent experiments. Primer sequences: (5′–3′): Foxp3 F (5′-CCTGGTTGTGAGAAGGTCTTCG-3′) and Foxp3 R (5′-TGCTCCAGAGACTGCACCACTT-3′); GAPDH F (5′-AAATGGTGAAGGTCGGTGTG-3′) and GAPDH R (5′-TGAAGGGGTCGTTGATGG-3′); T-bet F (5′-TCAACCAGCACCAGACAGAG-3′) and T-bet R (5′-ATCCTGTAATGGCTTGTGGG-3′); IL-17a F (5′-TCCAGAAGGCCCTCAGACTA-3′) and IL-17a R (5′-TGAGCTTCC CAGATCACAGA-3′); Rorc F (5′-GTGGAGTTTGCCAAGCGGCTTT-3′) and Rorc R (5′-CCTGCACATTCTGACTAGGACG-3′); IFN-γ F (5′-ACAGCAAGGCGAAAAAGGAT-3′) and IFN-γ R (5′-TGAGCTCATTGAATGCTTGG-3′); IL-10 F (5′-ATCGATTTC TCCCCTGTGAA-3′) and IL-10 R (5′-TGTCAAATTCATTCATGGCCT-3′) and IDO F (5′-GCAGACTGTGTCCTGGCAAACT-3′) and IDO R (5′-AGAGACGAGGAAGAAG CCCTTG-3′), from Invitrogen.

2.11. Statistical analysis

Data are represented as mean ± SD. Statistical differences between groups was carried out using the unpaired Student's *t*-test or the Two-way ANOVA, followed by the Bonferroni test, where appropriate. *P*-values of $\leqslant 0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software).

3. Results

3.1. GCN2 kinase plays an important role in EAE remission

Clinical analysis of GCN2 KO and WT mice subjected to EAE revealed that although no significant differences were found between the two groups from the onset until the peak of the disease (about 15 days post-immunization), significant differences were observed in the remission phase. In particular, from day 21 after immunization, GCN2 KO showed higher clinical scores (ranging between 3.0 and 3.5) while WT mice, as expected, begun to decrease the clinical symptoms (Fig. 1A). No significant difference was found in the disease incidence, which reached about 83% in WT and 88% in GCN2-KO mice (Fig. 1B).

In an attempt to establish a correlation between clinical signs and morphological changes in the CNS of EAE mice, histological sections of lumbar spinal cord were collected at day 15 (peak) and at day 21 (remission) after immunization and were analyzed in light microscopy. We noticed an inflammatory infiltration mainly composed by mononuclear leukocytes and predominantly localized in the submeningeal areas and around blood vessels of the white matter. The presence of the inflammatory cells was far more evident in GCN2 KO mice, especially at the remission phase of the disease (Fig. 2A and B). Likewise, although few morphological differences were noted between the groups at day 15 post-immunization (Supplemental Fig. 1), at day 21 post-immunization, GCN2 KO mice showed more pronounced demyelinating lesions, evidenced by luxol fast blue staining (Fig. 2C and D). Thus, histological analyses gave support to the clinical data showing that animals lacking the GCN2 kinase do not present the remission phase of EAE and this was associated with more intense inflammation within the spinal cord.

3.2. The absence of EAE remission in GCN2-deficient mice is associated with increased expression of Th1- and Th17-related cytokines and decreased expression of IL-10 and Foxp3 in the CNS

It has already been reported that in B6 mice, the partial remission following the peak of the disease is accompanied by a decrease in the frequency of CD4+ T effector cells expressing IFN-γ and/or IL-17 within the target organ (Korn et al., 2007; O'Connor et al., 2007). We then asked whether in GCN2 KO mice, the absence of the remission phase and the more intense presence of inflammatory cells would be associated with higher expression of cytokines related to Th1 and Th17 cells. The characterization of the cellular and the molecular profile of the CNS inflammation were performed by qPCR and flow cytometric analyses of the infiltrating leukocytes. First, looking at the expression of transcripts by qPCR, we found that, at the peak of the disease (15th day post-immunization), GCN2 KO mice already had higher levels of IL-17 and IFN-γ mRNA in sorted CNS-infiltrating CD3+CD4+ T cells than WT animals (Fig. 3A and B). Consistent with that, we also found higher levels of expression for the transcriptional factors Rorc (69% increase) and T-bet (12% increase) mRNA in CD4+ T cells recovered from the CNS of GCN2-deficient animals (Fig. 3C and D). At day 21 post-immunization (remission phase), we observed striking differences when comparing the expression of inflammatory cytokines in sorted infiltrating T cells from WT mice and GCN2 KO mice. The relative expression of IL-17 mRNA in GCN2 KO mice was about 4 times higher and the expression of IFN-γ, Rorc and T-bet were, respectively 80%, 68% and 79% higher than in WT mice (Fig. 3E–H). Flow cytometric analysis of CD4+ T cells infiltrating the target tissue corroborated qPCR data. In fact, at 21 days post-immunization, we also found an increased frequency of effector T cells secreting IL-17 and/or IFN-γ recovered from the CNS of GCN2-deficient mice (29.3%) as compared to that noticed in WT animals (18.3%) (Fig. 3I–K). Overall, the data suggests that GCN2 KO mice show more prevalent Th1 and Th17 CNS infiltration in EAE than WT do, in particular during the remission phase of the disease.

Increased frequency of regulatory T cells and the expression of anti-inflammatory cytokines such as IL-10 have been associated with EAE remission (Korn et al., 2007; O'Connor et al., 2007). Since GCN2-deficient mice developed a disease course in which the partial remission does not occur, we asked if the increased expression of Th1- and Th17-related cytokines would be associated with downregulation of Foxp3+ T cells and/or IL10-secreting cells. Because CD4+ T cells might not be the only source of IL-10 during EAE remission phase (Mann et al., 2007; Matsushita et al., 2008; Ray et al., 2011), we decided to evaluate the presence of transcripts for Foxp3 and IL-10 in the spinal cords instead of in infiltrating CD4+ T cells. Indeed, we found that, in the remission phase of EAE, GCN2 KO animals expressed lower levels of Foxp3 and IL-10 mRNA (about 55% less) (Fig. 4A and B). Confirming the analysis performed in CNS-infiltrating CD4+ T cells, we also observed higher levels of IL-17 mRNA (about a four fold increase) in total spinal cords of GCN2 KO animals as compared with WT mice (Supplemental Fig. 2). Once again, flow cytometric analyses reinforced the observations made in the qPCR experiments. Indeed, we observed lower frequency of $Foxp3^{GFP+}$ cells in gated CD3+CD4+ T lymphocytes infiltrating the CNS of GCN2 KO $Foxp3^{GFP}$ mice (18.7%) as compared to that in WT $Foxp3^{GFP}$ animals (30.4%)

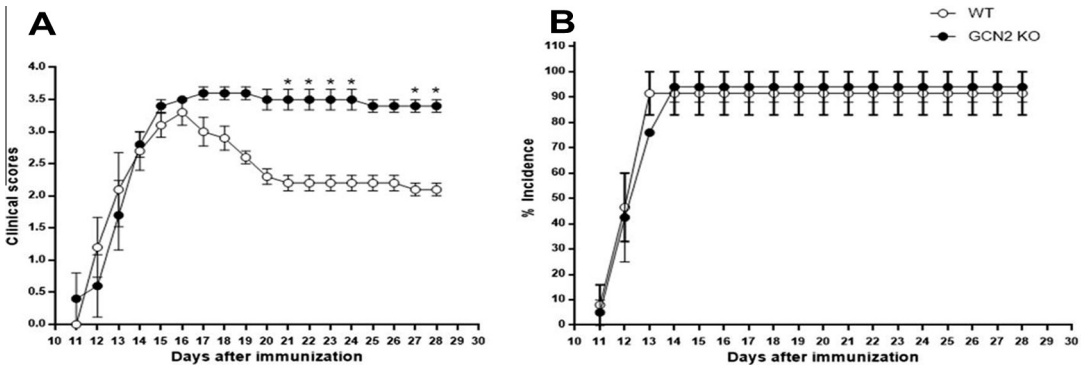

Fig. 1. GCN2 is important to the development of EAE remission. (A) EAE clinical course and (B) percentage of EAE incidence in GCN2 KO and WT mice (representative of five independent experiments performed with eight animals per group). Two-way Anova test. $^{*}p < 0.05$. Results are shown as mean ± standard deviation.

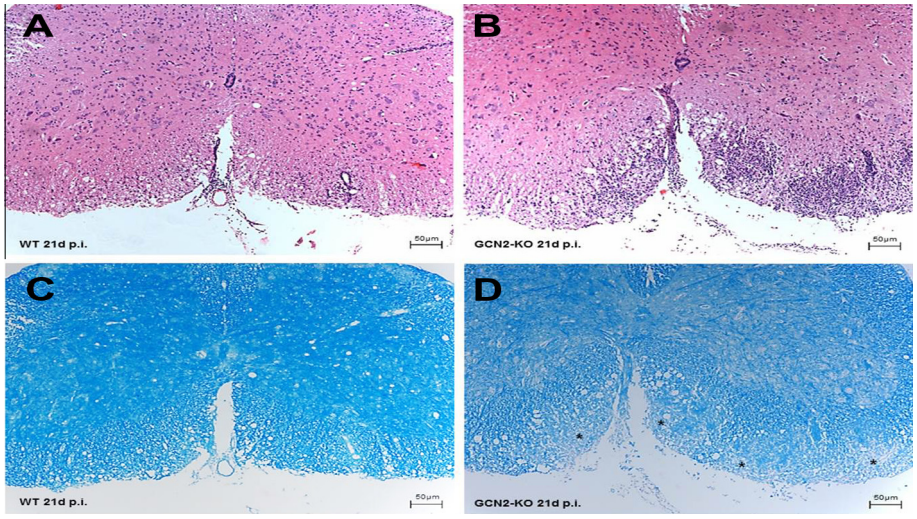

Fig. 2. GCN2-deficient animals display intense inflammatory lesions and demyelination 21 days after MOG immunization. Inflammatory infiltration in the spinal cord of (A) WT and (B) GCN2 KO mice 21 days post-immunization; demyelinating areas are indicated by (∗) in the spinal cord of (C) WT and (D) GCN2 KO mice 21 days post-immunization (representative of three independent experiments). H&E (A and B) and Luxol Fast Blue (C and D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4C–E). It is important to mention that lower frequency of Treg cells does not seem to be a general feature of GCN2 KO animals. In fact, we did not observe differences in the peripheral (spleen and lymph nodes) frequency of Foxp3^{GFP+} cells when comparing naïve WT and GCN2 KO mice (Supplemental Fig. 3).

Taken together, these results suggest that GCN2 KO animals exhibit higher pro-inflammatory responses within the CNS (mediated by the action of Th1 and Th17 cells) than WT animals, especially in the remission phase of EAE, which we demonstrated to be correlated with lower IL-10 expression and diminished presence of Foxp3+ regulatory T cells. In consequence, GCN2 KO mice develop EAE characterized by the absence of the remission phase of the disease and also more accentuated inflammatory and demyelinating lesions of the nervous tissue.

3.3. IDO expression within the CNS during EAE

The GCN2 kinase-dependent pathway is essential for cells to sense the lack of tryptophan – or the presence of its metabolite kynurenine – due to the activity of the enzyme indoleamine 2,3-dioxygenase (IDO). Among immune cells, IDO has been reported to be expressed by some antigen-presenting cells, in particular plasmacytoid dendritic cells (pDC), and to play an important role in immune regulation by promoting Foxp3+ T cell differentiation and by leading effector T cells to cell cycle arrest (Fallarino et al., 2006; Munn et al., 2005). We first sought to investigate the presence of pDC along the EAE course. We found that the frequency of pDC among mononuclear cells infiltrating the CNS in EAE mice is higher during the peak of the disease than during the remission phase (Fig. 5A–D). We also found that naïve WT animals do not express high levels of IDO within the CNS and that along the EAE course, IDO expression is much higher in the peak of the disease than during the remission phase, when it is closer to that observed in healthy animals. In fact, qPCR analysis revealed that the presence of IDO transcripts in the spinal cords of WT mice was about 18 times higher in the disease peak than in the remission phase of EAE (Fig. 5E). Thus, our data suggests that IDO expression within the CNS in EAE mice occurs during the period when inflammation is highest. During the remission phase, when inflammation has already diminished in WT mice but not in GCN2 KO animals, we observed a significantly higher presence of transcripts for IDO in the spinal cord from GCN2 KO mice as compared to that in the spinal cord from WT mice (Fig. 5F); though the levels of IDO expression verified during the remission phase in GCN2 KO mice are still lower than that observed at the peak of the disease. It is also important to mention that we did not find differences in IDO expression within the CNS between WT and GCN2 KO mice at the EAE peak (Supplemental Fig. 4).

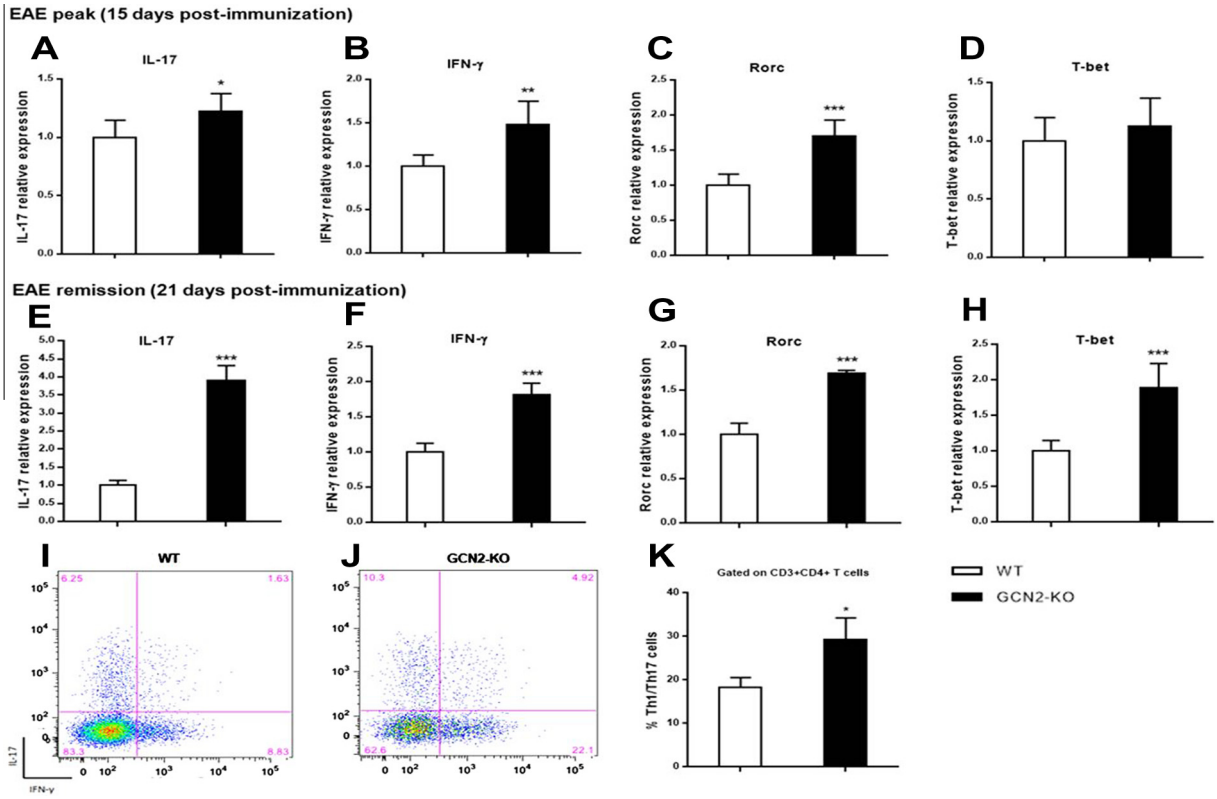

Fig. 3. Absence of the remission phase in GCN2-deficient animals is associated with higher Th1/Th17 response within the CNS. Relative expression of (A) IL-17, (B) IFN-γ, (C) Rorc and (D) T-bet in sorted CNS- infiltrating CD3+CD4+ T cells at the EAE peak (15th days post-immunization) and, respectively (E–H), at EAE remission stage (21st days post-immunization). Representative plots showing the frequency of IL-17-positive and IFN-γ-positive CNS infiltrating CD3+CD4+ T cells from (I) WT Foxp3^{GFP} and (J) GCN2 KO Foxp3^{GFP} mice at the remission phase (21 days post-immunization). (K) Statistical analysis of the plots shown in (I) and (J) (representative of four independent experiments). Student's *t*-test. $^{*}p < 0.05$. $^{**}p = 0.0076$. $^{***}p < 0.0001$. Results are shown as mean ± standard deviation.

We then hypothesized that inflammation itself, by inducing IDO expression, could be the trigger to promote regulatory T cell differentiation/expansion within the CNS and to initiate the remission phase during the course of EAE in WT mice. In contrast, GCN2 KO animals are not able to sense IDO activity and that would lead, at least in part, to a deficiency in the generation of regulatory T cells and the consequent absence of the remission phase during the course of the disease. In order to test this hypothesis we decided to assess the ability of WT IDO-expressing pDCs to convert either WT naïve T cells or GCN2-deficient naïve T cells into Foxp3+ regulatory T cells. Indeed, we found that splenic WT pDCs purified by cell sorting, when co-cultured with cell sorted anti-CD3 stimulated WT CD4+CD62L+Foxp3^{GFP-} naïve T cells, can induce the conversion of almost 8% of them into Foxp3^{GFP+} cells (Fig. 5G–I). In contrast, only 3.7% of GCN2-deficient naïve T cells could be converted into Foxp3GFP+ cells when co-cultured with WT splenic pDCs (Fig. 5G–I). Thus, the lack of the GCN2-dependent pathway in T cells jeopardizes the pDC-induced conversion of these cells into Foxp3+ regulatory T cells.

3.4. In vitro differentiation of naïve CD4+ T lymphocytes into effector or Foxp3+ regulatory cells

Finally, intending to evaluate if the results previously obtained from *in vivo* experiments could be related to an intrinsic ability of CD4+ T naïve cells from GCN2 KO mice to preferentially differentiate into effector Th1/Th17 cells than into regulatory T lymphocytes, sorted naïve Foxp3^{GFP-}CD4+CD62L+ T lymphocytes from WT Foxp3^{GFP} mice and from GCN2 KO Foxp3^{GFP} mice were activated *in vitro* along with different polarizing conditions. We observed that, when activated in Th17-driving milieu, about 30% of the naïve CD4+ T cells from GCN2 KO differentiated into IL-17-producing cells. In contrast, only 21% of the WT naïve CD4+ T cells became IL-17+ cells when activated in the same conditions (Fig. 6A–C). In accordance with the reciprocal development of Th17 and Foxp3+ regulatory T cells we observed the opposite when naïve CD4+ T cells were activated in the presence of TGF-beta. In this situation, while about 84% of the WT lymphocytes became Foxp3+ cells, we observed about 78% of the GCN2-deficient cells differentiating into regulatory T cells (Fig. 6D–F). No difference was noted in the differentiation of Th1 cells (Fig. 6G–I).

4. Discussion

Despite the advances obtained in recent years, much remains to be clarified about the etiological and pathological events governing inflammatory and demyelinating diseases of the CNS, such as MS. Due to the limitation found in the development of more extensive *ante-mortem* studies with humans, most of the information available is derived from animal models, such as murine EAE.

EAE is considered as a disease mediated primarily by the action of Th1 and Th17 lymphocytes, which release several pro-inflammatory cytokines and chemokines with consequent mobilization and activation of peripheral leukocytes to the CNS parenchyma (Batoulis et al., 2011; Becher and Segal, 2011; Duong et al., 1994; Komiyama et al., 2006; Langrish et al., 2005; Merrill et al., 1992; Park et al., 2005). Thus, while the peak of the disease is characterized by the prevalence of effector T cells (Th1 and Th17), the remission phase is accompanied by an enrichment of Treg cells, which become more frequent in the nervous tissue at this stage (Korn

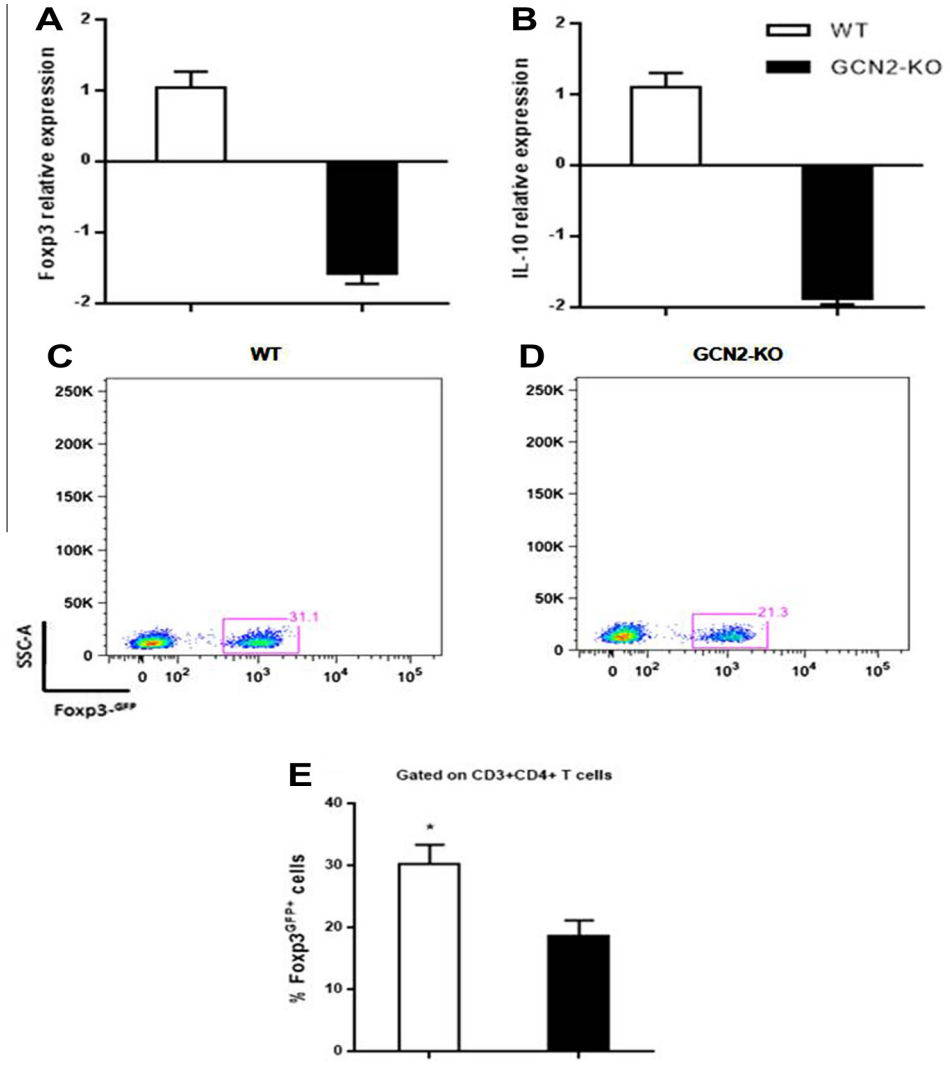

Fig. 4. Absence of the remission phase in GCN2-deficient animals is associated with lower frequency of Foxp3+ T cells within the CNS. Relative expression of (A) Foxp3 and (B) IL-10 in total extracts of spinal cords from WT and GCN2 KO mice at the EAE remission phase (21 days post-immunization). Representative plots showing the frequency of CNS-infiltrating $Foxp3^{GFP+}$ CD4+ T (Treg) cells from (C) WT $Foxp3^{GFP}$ and (D) GCN2 KO $Foxp3^{GFP}$ mice at the EAE remission phase (21 days post-immunization). (E) Statistical analysis of the plots shown in (C) and (D) (representative of four independent experiments). Student's *t*-test. $^{*}p = 0.017$. Results are shown as mean ± standard deviation.

et al., 2007). In fact, recent studies indicate a positive correlation between the presence of regulatory T cells and the regression of EAE symptoms (McGeachy et al., 2005; O'Connor et al., 2007), since their depletion inhibits the recovery of sick animals (McGeachy et al., 2005). Also in MS, the frequency and function of regulatory T cells appear to be inhibited, which could predispose the CNS to inflammation and subsequent demyelination (Haas et al., 2005; Venken et al., 2008; Viglietta et al., 2004).

The exact mechanisms by which the frequency of regulatory T cells increases within the target organ to promote EAE remission are still unclear. Herein we report that the GCN2 kinase plays an important role in triggering EAE remission, likely due to IDO-induced differentiation and/or expansion of Foxp3+ regulatory T cells.

GCN2 kinase plays a pivotal role in the cellular amino acid starvation response (AAR) and its activation occurs via the presence of amino acid uncharged tRNA, leading to decreased protein synthesis via phosphorylation of eIF2α (de Haro et al., 1996; Dever and Hinnebusch, 2005). This GCN2-dependent pathway exerts important functions controlling cell growth and/or differentiation (de Haro et al., 1996). In particular, considering immune cells, the GCN2-dependent pathway represents an essential sensor for the immunomodulatory effects elicited by the enzymatic activity of indoleamine 2,3-dioxygenase (IDO), which promotes tryptophan starvation due to its catabolism into kynurenine. In fact, it was already demonstrated that IDO-expressing plasmacytoid dendritic cells (pDCs) activate the GCN2 kinase pathway in T cells suppressing their proliferation upon TCR stimulation (Munn et al., 2005). Moreover, IDO induced profound anergy in responding WT T cells, but GCN2-knockout cells were refractory to IDO-induced anergy (Munn et al., 2005). In murine CD8+ T cells, IDO activity seems to result in GCN2 kinase-dependent down-regulation of the TCR zeta-chain. TCR zeta down-regulation could be demonstrated *in vivo* and was associated with an impaired cytotoxic effector function *in vitro* (Fallarino et al., 2006). Tryptophan catabolism has also been shown to induce apoptosis in thymocytes and Th1 cells (Fallarino et al., 2002). Recently, GCN2-dependent activation of the amino acid starvation response was found to inhibit mouse and human Th17 differentiation (Sundrud et al., 2009). Besides dampening the response of effector T cells, IDO-induced regulatory mechanisms also involve de novo generation/expansion of Foxp3+ regulatory T cells (Baban et al., 2009; Matteoli et al., 2010). Hence, it has been demonstrated that IDO-expressing DCs can induce Foxp3 expression when co-cultivated with Foxp3−CD4+CD25− T

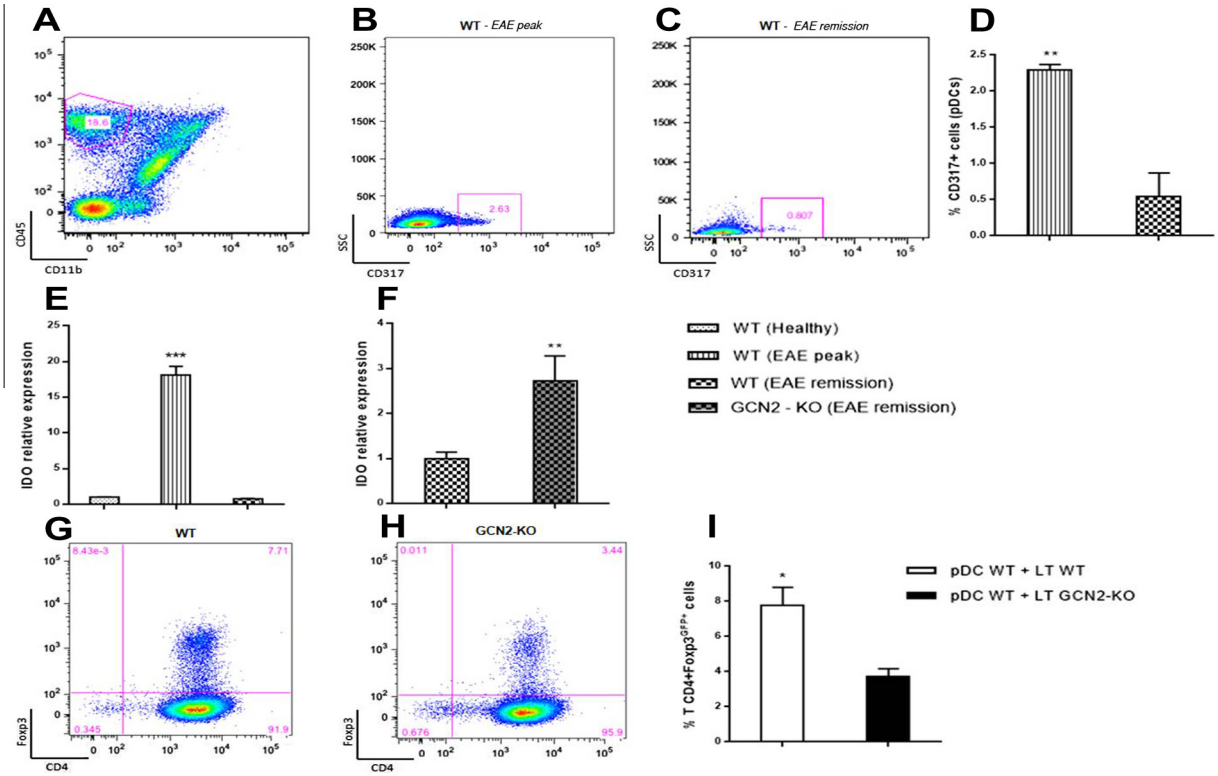

Fig. 5. IDO expression and pDC frequency within the CNS increase during EAE peak. Representative plots showing the frequency of pDCs (CD317+ cells) gated from (A) WT CD45+CD11b− infiltrating CNS cells at the EAE peak (B) and remission (C) stages (representative of three independent experiments). (D) Statistical analysis of representative data shown in (B) and (C). Student's *t*-test. $^{**}p = 0.0035$. (E) Relative expression of IDO in spinal cord extracts from healthy and sick WT mice at the EAE peak and remission stages. (F) Relative expression of IDO in spinal cord extracts from WT and GCN2 KO mice at the remission stage of EAE. Two-way ANOVA and Student's *t*-test. $^{**}p = 0.0071$. $^{***}p < 0.0001$. Representative plots showing the frequency of Foxp3^{GFP+} T cells differentiated *in vitro* from WT Foxp3−CD4+CD62L+ naïve T cells (G) or from GCN2 KO Foxp3−CD4+CD62L+ naïve T cells (H) co-cultured with WT pDCs. (I) Statistical analysis of representative data shown in (G) and (H) (representative of three independent experiments made in triplicate). Student's *t*-test. $^{*}p = 0.038$. All results are shown as mean ± standard deviation.

cells (Chen et al., 2008; Fallarino et al., 2006). Important to mention that the IDO-induced regulatory T cells indeed displayed suppressive activity in *in vitro* and *in vivo* assays (Fallarino et al., 2006). In a similar way, *in vitro* activation of lymphocytes in the presence of a tryptophan metabolite (3-HAA) promoted Treg differentiation (Chen et al., 2008). *In vivo*, IDO-expressing plasmacytoid DCs are also thought to create a profoundly suppressive microenvironment within tumor-draining lymph nodes via constitutive activation of regulatory T cells (Katz et al., 2008; Sharma et al., 2007). Finally, IDO-expressing DCs in the gut are also thought to play an important role in oral tolerance by promoting the differentiation of antigen-specific Treg cells (Matteoli et al., 2010).

Although many cell types can potentially express IDO, including non-hematopoietic cells such as fibroblasts and endothelial cells, among hematopoietic cells, the expression of IDO is often restricted to certain specific antigen-presenting cell subsets that appear specialized for upregulation of IDO in response to inflammatory stimuli. In particular, IDO expression has been well documented in pDCs. In fact, it has been demonstrated that in these cells, IDO is mainly induced by Toll-like receptor ligands (Baban et al., 2011; Volpi et al., 2012) and by cytokines such as IFN-γ (Mellor and Munn, 2004) and TNF-α (Kwidzinski et al., 2005).

In EAE, as well as in other inflammatory conditions, IFN-γ and TNF-α are highly expressed especially in the peak of the disease, when there is a predominance of effector T cells and other activated leukocytes within the CNS. Interestingly, in our experiments we observed that IDO expression in the CNS of WT animals is higher in the peak of the disease than in the remission phase, when IDO levels were comparable to that found in naïve mice. In addition, we observed higher frequency of pDCs in the CNS of WT animals also at the peak of the disease and a subsequent decrease in their frequency at the remission stage. In EAE, it was shown that depletion of pDCs during relapse phases of the disease resulted in exacerbation of clinical severity. Moreover, pDC depletion significantly enhanced CNS T cell activation, as well as IL-17 and IFN-γ production (Bailey-Bucktrout et al., 2008). In a similar way, another study showed that pDCs depletion 1 week after MOG immunization increased the clinical signs of the disease, indicating its immunomodulatory role at this stage (Isaksson et al., 2009). These observations may suggest that the increase in IDO expression at the peak of the disease, likely due to pro-inflammatory mediators that are present in the nervous tissue at this stage, initiates disease recovery by activating the GCN2 pathway, which in turn stimulates immunoregulatory responses involving apoptosis and/or anergy of effector T cells and induction and/or hyper-activation of Treg cells. In fact, we showed that, in contrast to what happens in WT mice in which the remission phase develops normally, animals that are unable to sense IDO activity due to the absence of the GCN2 kinase do not present the remission phase and this was associated to more pronounced inflammation, higher frequency of effector T cells, and lower frequency of regulatory T cells within the CNS. Although we have found a temporal association between IDO expression and frequency of pDCs at the peak of EAE in the CNS of WT animals, it is important to mention that other cells such as astrocytes (Guillemin et al., 2005; Suh et al., 2007) and microglia (Guillemin et al., 2005; O'Connor et al., 2009; Wang et al., 2010) can also express IDO within the inflamed CNS.

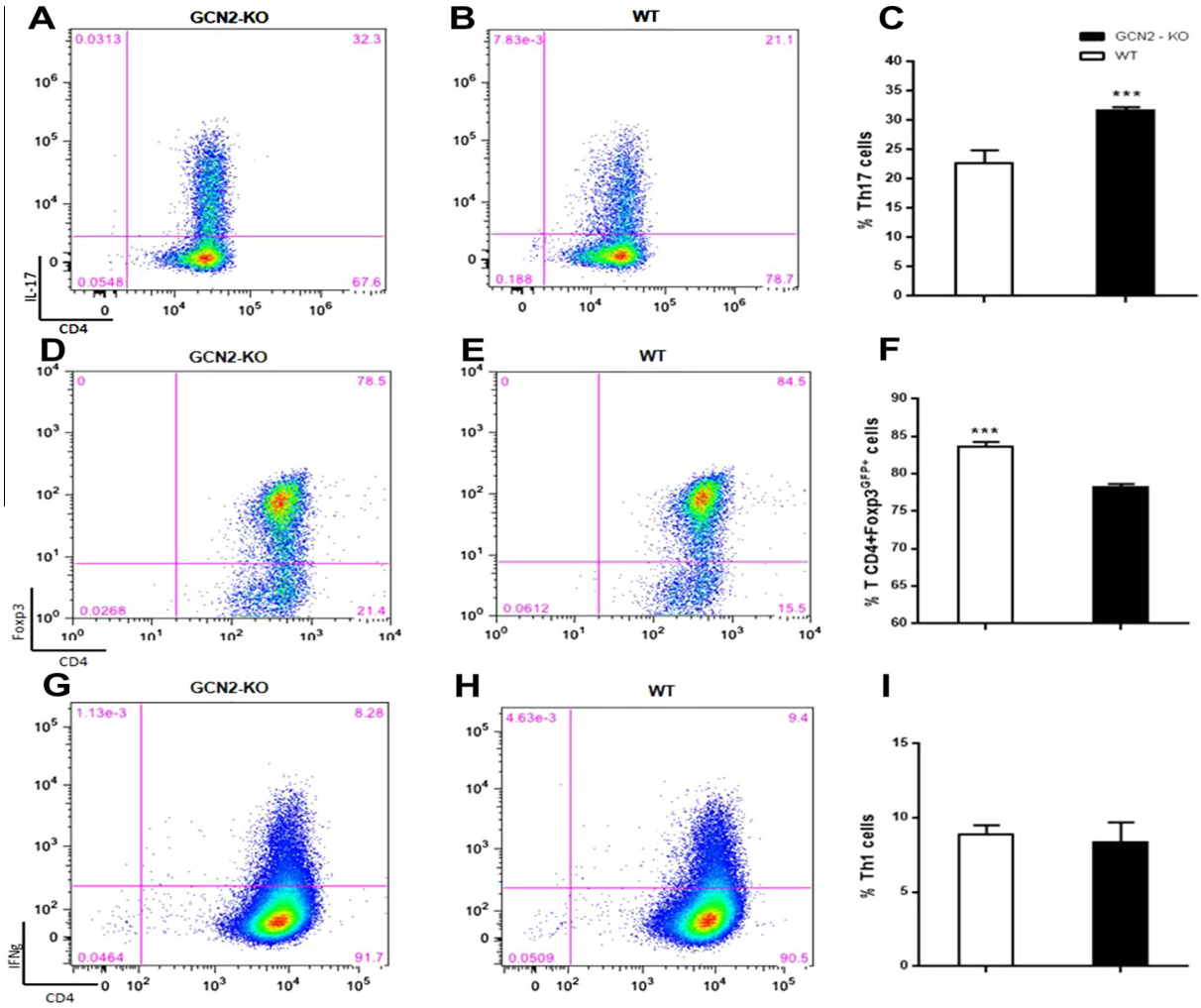

Fig. 6. GCN2-deficient naïve T cells are more prone to differentiate into Th17 cells. Frequency of Th17 cells differentiated *in vitro* from CD4+CD62L+ T naïve cells from (A) GCN2 KO and (B) WT mice. (C) Statistical analysis of the representative data shown in (A) and (B). Frequency of Treg ($Foxp3^{GFP+}$) cells differentiated *in vitro* from Foxp3−CD4+CD62L+ T cells from (D) GCN2 KO $Foxp3^{GFP}$ and (E) WT $Foxp3^{GFP}$ mice. (F) Statistical analysis of the representative data shown in (D) and (E). Frequency of Th1 cells differentiated *in vitro* from CD4+CD62L+ T cells from (G) GCN2 KO and (H) WT mice and (I) statistical analysis of the representative data shown in (G) and (H). Representative of three independent experiments made in triplicate. Student's *t*-test. $^{***}p < 0.0001$. Results are shown as mean ± standard deviation.

The immunosuppressive function of IDO in EAE has already been suggested in a study comparing disease severity in WT and IDO knockout mice (Yan et al., 2010). It was found that the absence of the enzyme promotes more severe symptoms of the disease associated with higher encephalitogenic response of Th1 and Th17 cells and decreased presence of Treg cells. Moreover, Sakurai et al. (2002) found that the treatment of animals subjected to passive EAE with 1-Methyl-DL-tryptophan (1-MT), which inhibits IDO enzymatic activity, augmented the mean maximal clinical score of the disease. Another study also found that IDO inhibition by systemic administration of 1-MT at clinical onset significantly exacerbated disease scores at the peak of the disease (Kwidzinski et al., 2005). Accordingly, treating mice with tryptophan metabolites has been shown to improve EAE symptoms (Platten et al., 2005; Yan et al., 2010). In agreement with our data, one of these studies also reported increased IDO activity within the CNS during the peak of the disease (Kwidzinski et al., 2005). Curiously, at late stages of the disease, we observed a significant increase in IDO expression in the spinal cords from GCN2 KO animals as compared to that in WT mice. This finding also gives support to our suggestion that upon ongoing inflammation (that is continuous in GCN2-deficient mice) IDO is probably stimulated to promote homeostatic balance. Thus, when sensing IDO activity fails because of the lack of GCN2, higher levels of inflammatory mediators are maintained, keeping the increased expression of IDO by the cells infiltrating the nervous tissue.

Consistently with the literature (Fallarino et al., 2006; Munn et al., 2005), the data from the *in vitro* experiments we carried out demonstrated that pDCs from WT mice promote more efficient differentiation of Treg cells from naïve T CD4+ lymphocytes than from GCN2-KO T cells, thus confirming that the pDCs-mediated differentiation of regulatory T cells is likely to depend on the ability of T cells to sense IDO activity. Unexpectedly, our *in vitro* experiments also demonstrated that naïve CD4+CD62L+ T cells from WT mice, when activated in the presence of TGF-β, differentiated more frequently into Treg cells than those from GCN2 KO animals. In accordance with the described reciprocal developmental pathways for the generation of Treg and Th17 cells (Bettelli et al., 2006), we also observed that the naïve T cells from GCN2 KO mice are more prone to differentiate into Th17 cells. Although this observation highlights a possible involvement of GCN2 in the dichotomy characterizing the differentiation of effector Th17 and regulatory T cells by mechanisms that were not elucidated, it might help explaining the higher expression of effector cytokines in the CNS of GCN2 KO animals already at EAE peak.

Altogether, our results suggest that the pro-inflammatory events mediated by Th1 and Th17 cells that lead animals to

develop the clinical symptoms characterizing the peak of the disease (especially the high expression of IFN-γ and TNF) promote the activation of IDO in APCs within the CNS, such as infiltrating pDCs. The enzymatic activity of IDO results in activation of GCN2 which in turn works as a trigger for immunomodulatory events (such as Treg cell differentiation and suppression of effector T cell responses), leading to a partial remission in the clinical signs of the disease. The absence of GCN2 prevents the suppressor functions of IDO, making the environment permissive to continued inflammation in EAE mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2013.12.012.

References

Ajami, B., Bennett, J.L., Krieger, C., McNagny, K.M., Rossi, F.M., 2011. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat. Neurosci. 14, 1142–1149.

Baban, B., Chandler, P.R., Sharma, M.D., Pihkala, J., Koni, P.A., Munn, D.H., Mellor, A.L., 2009. IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. J. Immunol. 183, 2475–2483.

Baban, B., Chandler, P.R., Johnson, B.A., Huang, L., Li, M., Sharpe, M.L., Francisco, L.M., Sharpe, A.H., Blazar, B.R., Munn, D.H., Mellor, A.L., 2011. Physiologic control of IDO competence in splenic dendritic cells. J. Immunol. 187, 2329–2335.

Bailey-Bucktrout, S.L., Caulkins, S.C., Goings, G., Fischer, J.A., Dzionek, A., Miller, S.D., 2008. Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. J. Immunol. 180, 6457–6461.

Batoulis, H., Recks, M.S., Addicks, K., Kuerten, S., 2011. Experimental autoimmune encephalomyelitis–achievements and prospective advances. APMIS 119, 819–830.

Becher, B., Segal, B.M., 2011. T(H)17 cytokines in autoimmune neuro-inflammation. Curr. Opin. Immunol. 23, 707–712.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238.

Chen, W., Liang, X., Peterson, A.J., Munn, D.H., Blazar, B.R., 2008. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J. Immunol. 181, 5396–5404.

de Haro, C., Méndez, R., Santoyo, J., 1996. The eIF-2alpha kinases and the control of protein synthesis. FASEB J. 10, 1378–1387.

Dever, T.E., Hinnebusch, A.G., 2005. GCN2 whets the appetite for amino acids. Mol. Cell 18, 141–142.

Duong, T.T., Finkelman, F.D., Singh, B., Strejan, G.H., 1994. Effect of anti-interferon-gamma monoclonal antibody treatment on the development of experimental allergic encephalomyelitis in resistant mouse strains. J. Neuroimmunol. 53, 101–107.

Fallarino, F., Grohmann, U., Vacca, C., Bianchi, R., Orabona, C., Spreca, A., Fioretti, M.C., Puccetti, P., 2002. T cell apoptosis by tryptophan catabolism. Cell Death Differ. 9, 1069–1077.

Fallarino, F., Grohmann, U., You, S., McGrath, B.C., Cavener, D.R., Vacca, C., Orabona, C., Bianchi, R., Belladonna, M.L., Volpi, C., Fioretti, M.C., Puccetti, P., 2006. Tryptophan catabolism generates autoimmune-preventive regulatory T cells. Transpl. Immunol. 17, 58–60.

Guillemin, G.J., Smythe, G., Takikawa, O., Brew, B.J., 2005. Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. Glia 49, 15–23.

Haas, J., Hug, A., Viehöver, A., Fritzsching, B., Falk, C.S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B., Storch-Hagenlocher, B., Krammer, P.H., Suri-Payer, E., Wildemann, B., 2005. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur. J. Immunol. 35, 3343–3352.

Isaksson, M., Ardesjö, B., Rönnblom, L., Kämpe, O., Lassmann, H., Eloranta, M.L., Lobell, A., 2009. Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE. Eur. J. Immunol. 39, 2925–2935.

Izikson, L., Klein, R.S., Luster, A.D., Weiner, H.L., 2002. Targeting monocyte recruitment in CNS autoimmune disease. Clin. Immunol. 103, 125–131.

Katz, J.B., Muller, A.J., Prendergast, G.C., 2008. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. Immunol. Rev. 222, 206–221.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K., Iwakura, Y., 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J. Immunol. 177, 566–573.

Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T.R., Bäckström, B.T., Sobel, R.A., Wucherpfennig, K.W., Strom, T.B., Oukka, M., Kuchroo, V.K., 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat. Med. 13, 423–431.

Kwidzinski, E., Bunse, J., Aktas, O., Richter, D., Mutlu, L., Zipp, F., Nitsch, R., Bechmann, I., 2005. Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. FASEB J. 19, 1347–1349.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., Cua, D.J., 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J. Exp. Med. 201, 233–240.

Mann, M.K., Maresz, K., Shriver, L.P., Tan, Y., Dittel, B.N., 2007. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. J. Immunol. 178, 3447–3456.

Matsushita, T., Yanaba, K., Bouaziz, J.D., Fujimoto, M., Tedder, T.F., 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J. Clin. Invest. 118, 3420–3430.

Matteoli, G., Mazzini, E., Iliev, I.D., Mileti, E., Fallarino, F., Puccetti, P., Chieppa, M., Rescigno, M., 2010. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut 59, 595–604.

McGeachy, M.J., Stephens, L.A., Anderton, S.M., 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J. Immunol. 175, 3025–3032.

Mellor, A.L., Munn, D.H., 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat. Rev. Immunol. 4, 762–774.

Merrill, J.E., Kono, D.H., Clayton, J., Ando, D.G., Hinton, D.R., Hofman, F.M., 1992. Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice. Proc. Natl. Acad. Sci. U. S. A. 89, 574–578.

Munn, D.H., Sharma, M.D., Baban, B., Harding, H.P., Zhang, Y., Ron, D., Mellor, A.L., 2005. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 22, 633–642.

Murphy, A.C., Lalor, S.J., Lynch, M.A., Mills, K.H., 2010. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. Brain Behav. Immun. 24, 641–651.

O'Connor, R.A., Malpass, K.H., Anderton, S.M., 2007. The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory T cells. J. Immunol. 179, 958–966.

O'Connor, J.C., André, C., Wang, Y., Lawson, M.A., Szegedi, S.S., Lestage, J., Castanon, N., Kelley, K.W., Dantzer, R., 2009. Interferon-gamma and tumor necrosis factor-alpha mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette–Guerin. J. Neurosci. 29, 4200–4209.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C., 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol. 6, 1133–1141.

Platten, M., Ho, P.P., Youssef, S., Fontoura, P., Garren, H., Hur, E.M., Gupta, R., Lee, L.Y., Kidd, B.A., Robinson, W.H., Sobel, R.A., Selley, M.L., Steinman, L., 2005. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science 310, 850–855.

Ray, A., Mann, M.K., Basu, S., Dittel, B.N., 2011. A case for regulatory B cells in controlling the severity of autoimmune-mediated inflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J. Neuroimmunol. 230, 1–9.

Roffé, M., Hajj, G.N., Azevedo, H.F., Alves, V.S., Castilho, B.A., 2013. IMPACT is a developmentally regulated protein in neurons that opposes the eukaryotic initiation factor 2α kinase GCN2 in the modulation of neurite outgrowth. J. Biol. Chem. 288, 10860–10869.

Sakurai, K., Zou, J.P., Tschetter, J.R., Ward, J.M., Shearer, G.M., 2002. Effect of indoleamine 2,3-dioxygenase on induction of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 129, 186–196.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.

Sharma, M.D., Baban, B., Chandler, P., Hou, D.Y., Singh, N., Yagita, H., Azuma, M., Blazar, B.R., Mellor, A.L., Munn, D.H., 2007. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J. Clin. Invest. 117, 2570–2582.

Suh, H.S., Zhao, M.L., Rivieccio, M., Choi, S., Connolly, E., Zhao, Y., Takikawa, O., Brosnan, C.F., Lee, S.C., 2007. Astrocyte indoleamine 2,3-dioxygenase is induced by the TLR3 ligand poly(I:C): mechanism of induction and role in antiviral response. J. Virol. 81, 9838–9850.

Sundrud, M.S., Koralov, S.B., Feuerer, M., Calado, D.P., Kozhaya, A.E., Rhule-Smith, A., Lefebvre, R.E., Unutmaz, D., Mazitschek, R., Waldner, H., Whitman, M., Keller, T., Rao, A., 2009. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science 324, 1334–1338.

Venken, K., Hellings, N., Thewissen, M., Somers, V., Hensen, K., Rummens, J.L., Medaer, R., Hupperts, R., Stinissen, P., 2008. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology 123, 79–89.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., Hafler, D.A., 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J. Exp. Med. 199, 971–979.

Volpi, C., Fallarino, F., Bianchi, R., Orabona, C., De Luca, A., Vacca, C., Romani, L., Gran, B., Grohmann, U., Puccetti, P., Belladonna, M.L., 2012. A GpC-rich oligonucleotide acts on plasmacytoid dendritic cells to promote immune suppression. J. Immunol. 189, 2283–2289.

Wang, Y., Lawson, M.A., Dantzer, R., Kelley, K.W., 2010. LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-gamma-independent manner by a JNK signaling pathway in primary murine microglia. Brain Behav. Immun. 24, 201–209.

Yan, Y., Zhang, G.X., Gran, B., Fallarino, F., Yu, S., Li, H., Cullimore, M.L., Rostami, A., Xu, H., 2010. IDO upregulates regulatory T cells via tryptophan catabolite and suppresses encephalitogenic T cell responses in experimental autoimmune encephalomyelitis. J. Immunol. 185, 5953–5961.