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T cell vaccination induces the elimination of EAE effector T cells: Analysis using GFP-transduced, encephalitogenic T cells☆

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ABSTRACT

T cell vaccination (TCV) with irradiated encephalitogenic T cells induces resistance to EAE. However, the fate of the encephalitogenic T cells in vivo following TCV has yet to be studied. Here we used anti-MBP encephalitogenic T cells that were transduced to express GFP to study the effects of TCV on these cells. In naïve rats or in control-vaccinated (Ova-GFP) rats injected i.v. with GFP-labeled effector cells, high numbers of effector T cells were found along with macrophages, CD8 T cells and Non-GFP CD4 cells in the spleens, parathymic lymph nodes (PTLN) and spinal cords. In contrast, the recipients that had been treated with TCV (anti-MBP T-cell lines) showed few if any GFP-labeled effector T cells throughout the disease (day 1–8) and their spinal cords were almost clear of macrophages, CD4 and CD8 cells. Splenocytes in the control groups secreted IFNγ in response to MBP and showed high numbers of IFNγ secreting CD4 and CD8 cells in their spinal cords at the disease peak. In the TCV-protected groups, splenocytes showed no reactivity to MBP but secreted IFNγ in response to irradiated encephalitogenic T cells – an anti-idiotypic response. Thus, TCV leads to a marked decrease in the numbers of effector T cells in the CNS and lymphoid organs, to a marked reduction in the Th1 cytokine producing cells in the CNS, and to the appearance of T cells responsive to the anti-MBP effector T cells.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) serves as a prototype for T cell-mediated autoimmune diseases [1] and has been used as a model for the pathogenesis of multiple sclerosis (MS) to help identify potential therapeutic candidates for this disease [2]. One such therapeutic modality is vaccination with antigen-activated or mitogen-activated attenuated encephalitogenic T cells called T cell vaccination (TCV) [3,4]. TCV is used to activate the immune system to recognize and neutralize disease-inducing effector T cells [5,6].

Effective treatment using TCV [4] was demonstrated in various experimental autoimmune diseases in rodents such as in adjuvant arthritis [7], experimental autoimmune thyroiditis [8], collagen-induced arthritis [9], experimental autoimmune uveitis [10], lupus [11], and type-1 diabetes [12]. TCV has been applied successfully to humans to treat MS [4,5], rheumatoid arthritis [13], and lupus [14]. Thus, the protective mechanisms induced by TCV have clinical relevance.

TCV was shown to activate anti-idiotypic T cells that are able to recognize the encephalitogenic T cells by unique TCR-peptide sequences [12,15]. Regulatory anti-idiotypic CD4 T cells [16] were able to shift the response from pathologic anti-myelin Th_1 responses to protective Th_2 responses [17] and to provide help to anti-idiotypic CD8 T cells, which appear to be the major suppressors of the autoimmune effector T cells [18]. $CD8^+$ suppressive T cells, induced following TCV in rats, were shown to specifically suppress the response of anti-MBP T cells to their cognate antigen [15], and to lyse them [18].

The CD8 regulator cells were reported to recognize TCR peptides presented on the MHC class Ib molecule, Qa-1, expressed on activated CD4 T cells [19,20]. In human multiple sclerosis patients, CD8

Abbreviations: ATCC, American Type Culture Collection; NRS, Normal Rat Serum; RBC, Red Blood Cells; TCGF, T cell Growth Factor; TBI, Total Body Irradiation; Ova, Ovalbumin; EAE, experimental autoimmune encephalomyelitis; TCV, T-cell vaccination; MBP/GBP, guinea-pig MBP (myelin basic protein); RBP, rat MBP; BBP, Bovine MBP; HBP, Human MBP.

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regulatory T cells isolated from the CSF exhibited HLA-E (Qa-1 human orthologue) restricted killing of MBP or MOG-specific $CD4^+$ targets [21]. However, TCR-peptide presentation on the rat Qa-1 orthologue has yet to be shown.

Another type of regulation is anti-ergotypic regulation. Ergotopes are molecules expressed on T cells upon activation, such as a peptide from the alpha chain of the IL-2 receptor (CD25); transfer of a T cell line responsive to the CD25 peptide was found to down-regulate EAE [22]. The same effect could be achieved by vaccination with antigen non-specific, activated T cells [23].

Several studies showed that TCV induces cytolytic anti-idiotypic T cells that might eliminate the autoimmune effector T cells [18]. Limiting dilution studies in MS subjects treated with TCV [24] or in rodents treated with TCR-peptide vaccination [25] have demonstrated a reduction in the T cells responding to myelin antigens. However, a decline in the frequency of T cells responding by proliferation to myelin antigens cannot tell us whether the actual effector cells have been eliminated or not; they may have been rendered anergic [26] or sequestered in sites that were not sampled as in animals that have spontaneously recovered from EAE and have acquired resistance to reinduction of the disease [27]. Therefore, the ultimate fate of the encephalitogenic effector T cells induced by TCV remains unknown.

In the present study, we followed the fate of encephalitogenic T cells lines in rats protected from EAE by TCV. The T cell lines were transduced with GFP expression cassettes to follow their fate at the single-cell level.

Various cell-marking techniques have been used to follow T cells, most of these methods have used chemical or physical means to mark the cells, but such marking can affect cell functions [28]. An alternative way to mark T cells is to transduce them with GFP expression cassettes [29,30]. GFP-marking of T cells allows following the cells in various biological assays, but does not adversely affect their biological functions. This technique was used to describe the dynamics of cell homing and function throughout the course of EAE [30]. We sampled the spinal cord (a target organ), the spleen and the parathymic lymph nodes. These representative sites exhibited the greatest number of GFP cells following transfer of GFP-transduced anti-MBP T-cells to Lewis rats [30]. The sites were sampled 24 h to 8 d following T-cell injection – before these time points cells GFP T-cells could not be detected in any location [30] and after these time points adoptively transferred cells decreased in numbers or disappeared from the sampled tissues [30].

2. Materials and methods

2.1. Animals

Female Lewis rats, aged 6–8 weeks were obtained from Harlan Israel and were maintained in an SPF environment. All animal experiments were conducted following institutional guidelines and supervision by institutional animal committee.

2.2. Raising and maintaining T-cell lines

T-cell lines specific for MBP were established and maintained using a standard protocol [1]: Briefly, lymphocytes from the draining lymph nodes of CFA/antigen immunized rats were isolated on day 12 post-immunization. The lymphocytes were stimulated *in vitro* (at 5×10^6/ml) for 72 h with 20 μg/ml of MBP (Sigma, Saint Louis, Missouri, USA). Stimulation medium contained DMEM, 1% syngeneic normal serum, 2 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 1% nonessential amino acids. Following a three-day cycle of stimulation, the cells were collected and transferred for a four-day rest in propagation medium containing DMEM (Biological Industries, Kibbutz Beit Haemek, Israel), 10% Fetal calf serum (FCS) (Hyclone, Thermo Scientific, Logan, UT, USA) 2 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 1% nonessential amino acids and 10% of TCGF medium (supernatant of rat splenocytes stimulated for two days with 2 μg/ml of ConA).

In the next cycles of stimulation, gamma-irradiated (5000 rad) syngeneic rat thymocytes (at 10^7 cells/ml) were used as antigen-presenting cells (APC). The APC were added to resting T cells (at 5×10^5 cells per ml) for three days of stimulation with 10 μg/ml of antigen.

2.3. GFP transduction of T-cell lines

GFP transduction of rat lymphocytes was conducted on primary T lymphocytes isolated from lymph nodes, following the method described by Flügel et al. [29].

T cells were transduced by cocultivation of packaging cells, GP + E86, producing replication-deficient MoMLV-derivative pLXSN-GFP-retrovirus (termed GP + E86-GFP cells). The pLXSN-GFP retroviruses also encoded Neomycin resistance gene, which allows for selection of transduced cells with G418 (Sigma).

2.4. T-cell lines

The following $CD4^+$ T-cell lines were raised or received, and used in both in vivo and *in vitro* experiments.

BP12 is a Lewis ($RT1^l$) rat T-cell line recognizing gpMBP and specifically the immunodominant 71–90 peptide from rat MBP. The line was raised from cells obtained from draining LN of CFA/gpMBP injected Lewis rats. This line represents a vaccine that can induce both anti-idiotypic and anti-ergotypic responses.

BP12-GFP is a Lewis rat T-cell line recognizing gpMBP. The line was raised from the same primary culture used to raise the BP12 line. The line was transduced with a retroviral vector encoding for Neomycin resistance and for GFP, an exogenous immunogenic protein [31]. This line represents a vaccine expressing both the TCR epitopes of the encephalitogenic transferred cells (anti-idiotypic), while it also expresses two exogenous antigens (Neomycin resistance, and GFP) that represent targets for a classical immune reaction against foreign antigens [31]. The line can also induce anti-ergotypic responses. The line was used both for vaccination of rats and for induction of EAE via i.v. injection.

Ova-GFP is a Lewis rat T-cell line recognizing Ovalbumin (Sigma), the line was transduced with the retroviral vector encoding for Neomycin resistance and for GFP. The line represents a target to which anti-foreign-antigen (GFP, neomycin resistance) and anti-ergotypic responses might be directed.

2.5. Vaccination with irradiated T cells and induction of EAE

Rats were vaccinated three times at weekly intervals with activated T cells that were irradiated at 3000 cGy, and injected s.c. in the flank at 10^7 cells in 100 μl PBS.

For the adoptive transfer of EAE, rats were injected i.v. 7–10 days after the last vaccination with 5×10^6 antigen stimulated T cells (BP12-GFP). Signs of EAE were scored using the disability scale in which 0 = absence of clinical signs; 0.5 = flaccid tail; 1 = loss of motor control in the whole tail; 1.5 = hindquarter weakness; 2 = paralysis of hind legs; 3 = half body paralysis; 4 = paralysis in front and back legs; 5 = total paralysis including neck movement; and 6 = death caused by EAE.

2.6. Isolation of T cells from spinal cords

Spinal cords were surgically removed from rats, dissected into pieces and transferred in RPMI medium without serum to a fine cell

mesh. The pieces were gently pressed through the mesh using a syringe plunger and collected to a serum-coated 50 ml tube in a total of 12.5 ml RPMI medium. Isotonic percoll – 5.8 ml (9 parts of percoll) (Pharmacia 17-0891-01 1.130 g/ml) to one part of PBS (×10) was added to the cells and mixed gently. The mixture was then gently underlayed with 5 ml percoll (7 parts of isotonic percoll to 3.9 parts PBS) and centrifuged for 30′ at 2800 rpm, 20 °C, brake off. Tissue remnants found at the top of the tube were gently aspirated, and the white interface was collected into a serum-coated 50 ml tube. RPMI-FCS was added up to 50 ml and the cells were collected by centrifugation at 1400 rpm, 1–2 times. All procedures were performed at 4 °C.

2.7. FACS analysis and antibodies

FACS analysis was used to determine cell surface markers and intra-cellular cytokine expression. Cells were stained according to standard staining procedures. The cells were monitored by FACSort or FACS Vantage (Becton Dickinson, Franklin Lakes, NJ, USA). The following mouse antibodies were used for FACS analysis: Anti-rat leucocyte sialoglycoprotein-CD43: W3/13 ascites fluid staining all rat leukocytes; Anti-rat CD8: OX-8 ascites fluid; anti-rat CD4: W3/25 ascites fluid; anti-rat IL-2 receptor α-chain (CD25) (BioLegend), anti-rat OX-40 Receptor (CD134) (Serotec); anti-Rat MHC class II RT1D monomorphic: OX-17 (Serotec); anti-rat macrophage marker ED-1: CD68 (Serotec). Indirect staining of cells was done using donkey anti-mouse Cy5 antibody (Jackson).

2.8. FACS detection of GFP-labeled cells

Spleens, PTLNs, blood or cells from spinal cords were collected from rats injected with GFP-labeled T cells. Cell suspensions from these organs were analyzed by flow cytometry according to originators guidelines [29]. Cells that appeared $FL1^{high}/FL2^{low}$ outside the area of autofluorescence depicted on FL1×FL2 axis were considered GFP-positive cells. Cells obtained from rats that had not been injected with GFP-labeled T cells (termed 'untreated') were used as negative controls in each of these experiments. Rats that were not treated by TCV, but were injected with GFP-labeled T cells were termed 'naïve injected'.

2.9. Restimulation of splenocytes ex-vivo

Splenocytes obtained from rats were restimulated in stimulation medium containing 10 μg/ml gpMBP and 0.4 mg/ml G418. After 3 days of stimulation, cells were washed and transferred to G418-containing propagation medium for 4–9 days. The cells were then assayed by FACS for GFP expression.

2.10. Immunohistochemistry

Immunohistochemistry was conducted on 4% PFA-fixated tissues. PFA fixation of tissues was done for at least 24 h. Then 30% sucrose was added 1:1 V:V for cryoprotection. The organs were then frozen and cut into 14 μm sections in a floating section microtome, slices were kept in PBS-0.1% Azid. For blocking, tissues were blocked 1 h RT in PBS (without Ca or Mg) with 20% Normal donkey serum (Jackson) and 1% Normal rat serum (NRS). For antigen staining, sections were incubated with different mouse monoclonal antibodies overnight at 4 °C. The antibodies were diluted in PBS containing 0.5% bovine serum albumin (BSA) and 0.09% Azid. Antibodies used were: ED-1 (1:500) for rat macrophages, W3/13 (1:500) for total rat T cells, OX-8 (1:250) for rat CD8 cells, the W3/25 anti-CD4 antibody could be used for FACS, but was not suitable for staining fixed tissue. Sections were washed twice for 10 min with $PBS^{-/-}$ and incubated with CY3-labeled donkey anti-mouse antiserum (1:5000) (Jackson) for 3 h RT. Counterstaining of cell nuclei was done with Hoechst 33342 (Molecular probes). Slices were transferred to Superfrost Plus slides (Menzel Glaser, Germany) and mounted with AquaPolyMount (Polysciences, USA).

2.11. Intra-cellular staining for cytokines

Monitoring of intra-cellular cytokine secretion was done as described elsewhere [32]. Briefly, cells obtained from rat spinal cords were incubated in stimulation medium containing PMA and ionomycin (5 μg/ml and 1 μM respectively). After 4 h, GolgiPlug© (BD) at 1:1000 from original stock, was added to the cultures. After 3–4 h with GolgiPlug© the cells were fixed in 4% PFA, at 4 °C for 20′ and washed twice with FACS buffer (PBS, BSA 0.5%, Azid 0.01%). The intra-cellular staining was done using the BD intra-cellular staining kit following the manufacturer's protocols. Antibodies used were anti-IFN-γ, anti-IL4 and an IgG isotype control, all PE-coupled.

Percent positive cells (Δ cytokine positive cells) were calculated by subtracting the percents of cytokine secreting cells found in the $FL2^{high}/FL1^{low}$ region with the background staining with isotype-control-PE antibody found in the same defined region in matching samples.

2.12. Co-culture proliferation and ELISA assays for splenocytes and irradiated T cells

Various antigens or irradiated T cells (3000 cGy) served as stimulators in proliferation assays. Rat splenocytes and antigens or irradiated cells were dispensed into u-shaped 96-well culture plates. Two days later, 80 μl of supernatant was taken from each well to be used in an ELISA assay, determining IFNγ, IL-10 or TNF-α concentrations using standard manufacturer protocols (BD-OptEIA). The wells from which supernatant was taken for ELISA were then pulsed with radioactive H^3 thymidine to determine cell proliferation.

In experiments where T cells served as stimulators, the background IFNγ secretion of the irradiated T cells alone was subtracted from the IFNγ secretion of effector T cells co-cultured with irradiated stimulator T cells.

3. Results

3.1. Clinical manifestations of EAE

T-cell vaccination was performed by immunizing rats s.c. three times at weekly intervals with irradiated antigen-activated T-cells. Two T-cell lines responding to MBP were used (the BP12 line and the GFP transfected BP-12-GFP line), and one control line was used – Ova-GFP, a GFP transfected line responding to Ova. Seven to ten days after the last vaccination the rats were injected i.v. with activated BP12-GFP cells and followed for clinical manifestations of EAE.

Fig. 1 depicts the effects of TCV on clinical EAE. The figure demonstrates that TCV with either the anti-MBP T cell lines BP12 or the BP12-GFP lines abrogated completely the development of clinical signs of EAE. In contrast, TCV with the Ova-GFP line had insignificant effects on the development of EAE in comparison to the control (naïve) rats injected with BP12-GFP.

3.2. TCV reduces the number of encephalitogenic T cells in a time-dependent manner

Flügel et al. have reported that the cells mediating EAE have a time-dictated migration pattern exhibited by a strong surge of

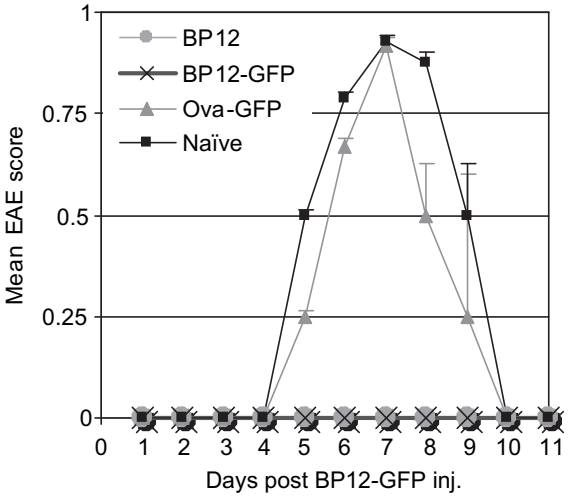

Fig. 1. The effect of TCV on clinical manifestations of EAE: Rats were immunized three times with irradiated activated cells from the BP12, BP12-GFP, and Ova-GFP lines at weekly intervals or were not treated (naïve). All groups were injected i.v. with encephalitogenic BP12-GFP cells a week or ten days after the last vaccination. Rats were followed for physiologic manifestations of EAE; Symptoms were scored using the standard disability scale. Results represent means of EAE scores depicted with standard error bars. The figure represents summary of two identical experiments with $N = 4–5$ rats per group in each experiment. Similar results were observed in four independent experiments.

cells into the parathymic lymph node (PTLN) peaking at day 3, to the spleen, peaking on day 4; the entry into the spinal cord starts between 36 and 60 h post T-cell transfer, and peaks with up to 10% of the total cells found in the CNS that are effector T cells by days 3–4 post-transfer [30].

To analyze homing and distribution of BP12-GFP cells in spleens, PTLNs and spinal cords, we collected organs from rats 1, 2, 4, 7 and 8 days post BP12-GFP transfer. Fig. 2A depicts representative FACS results of rat tissues taken at day 7, which represents the peak of clinical EAE in these sets of experiments. On day 7, plentiful cells appeared in the spinal cords of the naïve group and the Ova-GFP vaccinated group; approximately 1/70 cells of equal size and granularity were GFP-expressing cells. Lower, but comparable numbers of cells were found in the spleens and PTLN of the Ova-GFP and naïve groups on the same day. In contrast, no cells or very few cells appeared in spleen, PTLN and spinal cords of both the BP12 and BP12-GFP treated groups (usually under 3 cells per million).

Similarly, organs from rats were harvested on days 1, 2, 4, 7 and 8 following BP12-GFP transfer. Fig. 2B and C depicts the time-dependent cell migration into spleen (2B), and spinal cord (2C). The effect of TCV on the encephalitogenic T cells is noticeable in the spleen as early as day 2 post-injection and is almost full by day 7. In the PTLN, the disappearance of BP12-GFP cells is almost complete by day 7 (not depicted), while the number of cells in the spinal cord declines from low numbers of 20–30 cells per 10^5 cells on day 2 to an average of 2 cells per 10^5 by day 7.

Some reduction of GFP cell frequency was observed when comparing the Ova-GFP treated to the naïve-injected group. This reduction, although having minor effects clinically, could be attributed to anti-ergotypic, or anti-foreign (e.g. GFP) responses.

The complete disappearance of GFP cells from primary and secondary immune organs and from the spinal cords was found in the majority, but not in all of the animals in the TCV-protected groups (complete loss of cells in the spinal cord is depicted in Fig. 2A). Notwithstanding, at no stage were clinical signs observed in any of the TCV-protected animals.

3.3. GFP-expressing T cells can be re-isolated from the spleens of protected and non-protected rats

To assess whether any residual BP12-GFP cells remained in the spleens of TCV-treated rats, spleens of rats were harvested on different days following BP12-GFP injection and the splenocytes were re-cultured with MBP under Neomycin selection. Table 1 shows the comparative amounts of GFP-labeled cells found following the restimulation. GFP-positive T cells could be isolated and grown from spleens taken from all groups two or four days following BP12-GFP transfer, albeit in considerably smaller amounts in the TCV-protected groups. Sparse numbers of GFP-positive T cells could be re-isolated and grown from a few of the spleens harvested seven days post-transfer from rats in the TCV-protected groups. This is to be compared with the ample cells that were re-isolated and grown in the Ova-treated and naïve groups. As before, some minor effect of reduction in the re-isolated GFP-positive cells was observed also in the Ova-GFP treated group.

3.4. Localization of inflammatory cells detected by immunohistochemistry

We conducted histological analysis of CNS tissue to determine the exact localization of cells in the CNS of TCV treated and untreated rats. Spinal cords and brains were isolated, fixed and stained with W3/13 (CD43-total T cells), OX-8, (CD8 Cytotoxic T cells), and ED-1 (CD68-Macrophages). Cells were counterstained with Hoechst 33342 to mark the cell nucleus. Fig. 3 displays slices of spinal cord excised at the peak of disease from various TCV-treated groups and from a non-vaccinated naïve-injected group. Cells with a blue center and a red/yellow circumference are nucleated cells (blue) positively stained with the respective antibody (red).

Cells staining positive to any of the antibodies were very rarely found in slices of spinal cords or brains of rats treated by TCV with BP12 or BP12-GFP (Fig. 3A, E, I and B, F, J). In contrast, cells in the Ova-GFP treated (Fig. 3C, G, and K) and in the naïve group (Fig. 3D, H and L) were plentiful, and were found more frequently in the pia mater surrounding the spinal cord and in the white matter, especially in the funiculus ventralis and dorsalis. Cells could also be seen, in considerably smaller numbers, entering the grey area (the posterior and anterior horns) (data not shown). No major histological differences were apparent between the Ova-GFP treated and the naïve-injected group.

3.5. TCV reduces the numbers of inflammatory cells in the CNS

Along with massive reduction in the numbers of encephalitogenic GFP-expressing T cells infiltrating the CNS, we also observed a reduction in the numbers of inflammatory cells generally in the CNS following TCV. To check for the presence of inflammatory cellular markers, cells were isolated from spinal cords at different time points of the disease and were stained with the following antibodies: Rat MHC class II RT1.D, CD8, CD4, Total leukocyte/T-cell-CD43, the IL-2 receptor α-chain-CD25 and the OX-40 receptor-CD134. The cells were analyzed by FACS.

The results depicted in Fig. 4 show a distinctive infiltrate of T cells, both CD8 and CD4, in the spinal cords of the Ova-GFP treated group and the naïve-injected group. Very few, if any T cells were observed in the spinal cords of the BP12 or BP12-GFP treated groups. The Ova-GFP treated group and the naïve-injected group showed marked expression of MHC class II in cells found in the spinal cord. These cells are likely to be either activated T cells [33] or resident antigen-presenting cells [34]. Lower, though detectable expression of the CD25 T cell activation marker [32] and the OX-40 receptor were found in the non-protected groups. The OX-40 receptor is a marker

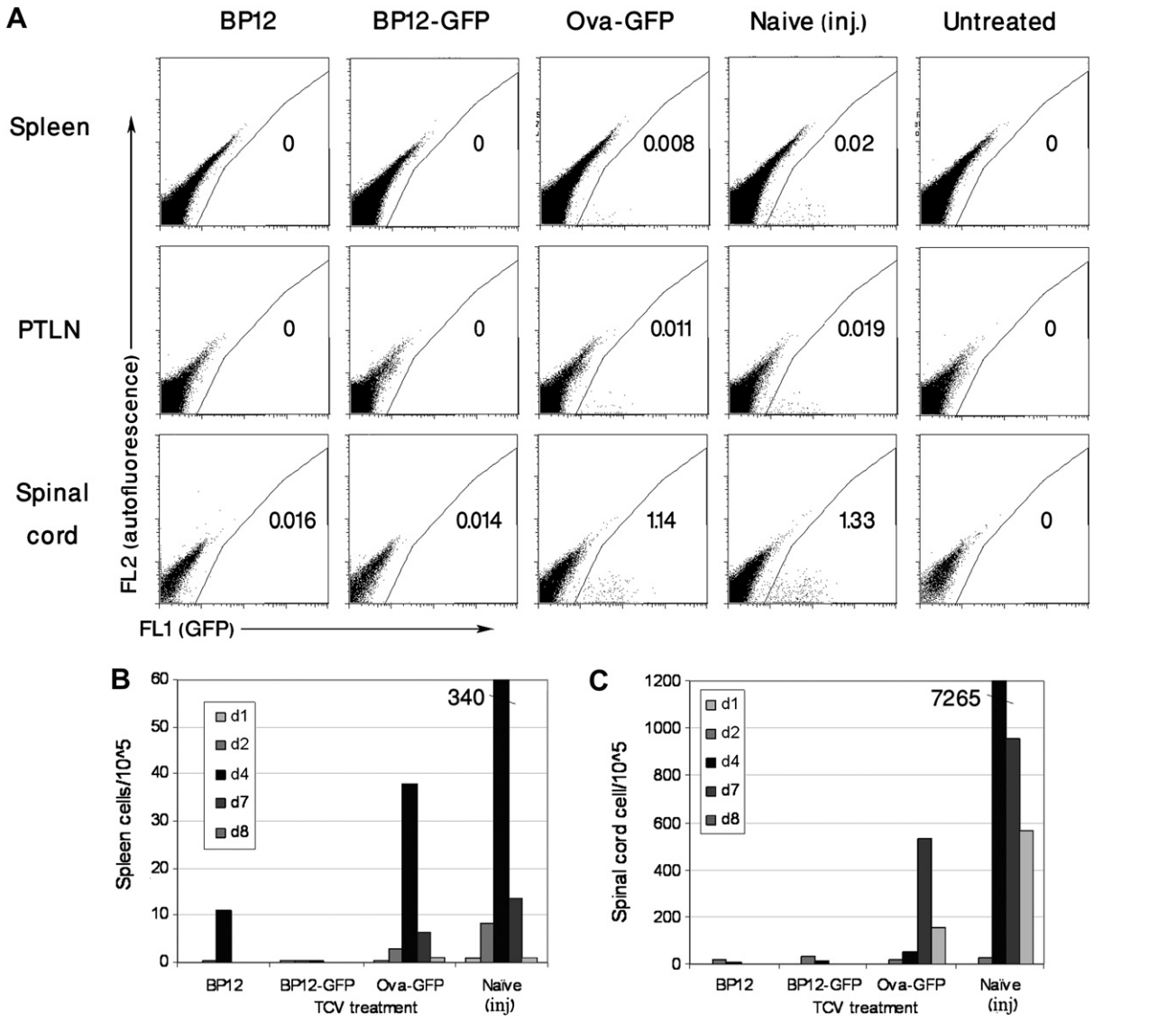

Fig. 2. Cytofluorometric analysis for quantification of BP12-GFP cells in rat organs: (A) Rats vaccinated either with BP12, BP12-GFP or Ova-GFP T-cells or not treated (Naïve-inj.) were injected i.v. with BP12-GFP cells. Spleens, PTLN and spinal cords were collected on day 7 post-transfer and analyzed by flow cytometry. Numbers in dot plots are the percent of BP12-GFP cells among lymphocytes of similar size and granularity found in the respective organ (~90% of spleen cells, ~80% of PTLN cells, and ~50% of Spinal cord cells). The data are representative of organs from 3 or more rats taken on day 7 in three similar experiments. Organs from an uninjected rat (Untreated) were used as controls. (B and C) Presence of GFP-labeled cells in spleens (B) and spinal cords (C) determined at days 1, 2, 4, 7 and 8 (d1–d8) following BP12-GFP injection. Numbers shown are mean number of cells per 10^5 cells with similar size and granularity as lymphocytes. The data are representative of 2–3 rats per point from two identical experiments. In d4, data from one rat are depicted. The data are representative of three similar experiments.

expressed on activated T cells and was found to be an important component for the development of strong Th1-mediated EAE [35]. Expression of these markers and the elevated levels of MHC-II in the TCV non-protected groups mark an active inflammatory process.

Table 1
Cytofluorometric analysis for assessment of BP12-GFP cells in restimulated splenocyte cultures: Splenocytes were collected from rats on days 2, 4 or 7 post-transfer and restimulated with MBP in Neomycin containing medium. The cells were then transferred to resting medium for 6–11 days. The cells were then analyzed by flow cytometry for GFP expression. The number of + signs represent the comparative number of cells found in the cultures after the restimulation. +/− indicates that no cells were found in some rats while in other rats only a small number of T cells were found. The data are representative of two similar experiments.

	BP12	BP12-GFP	Ova-GFP	Naïve-inj.	Untreated
Day-2	+	+	++	+++	−
Day-4	+	+	++	+++	
Day-7	−/+	−/+	+	++	

In contrast, TCV with either BP12 or BP12-GFP lines prevented accumulation in the CNS of the cells associated with the inflammatory process as well as most, if not all of the encephalitogenic T cells. A few of the rats in the TCV-protected groups exhibited a small number of CD4 cells in their spinal cords but showed no signs of clinical EAE (not shown).

3.6. TCV reduces the numbers of cells in the spinal cord expressing IFNγ

To determine cytokine secretion by cells entering the CNS, cells were isolated from rat spinal cords and stained intracellularly for IFNγ or IL-4 and read by FACS. Fig. 5 depicts the production of IFNγ and IL-4 by cells stimulated with PMA and ionomycin found at day 7 post-transfer (the disease peak). Activation by mitogenic PMA and ionomycin reveals cells capable of producing IFNγ or IL-4.

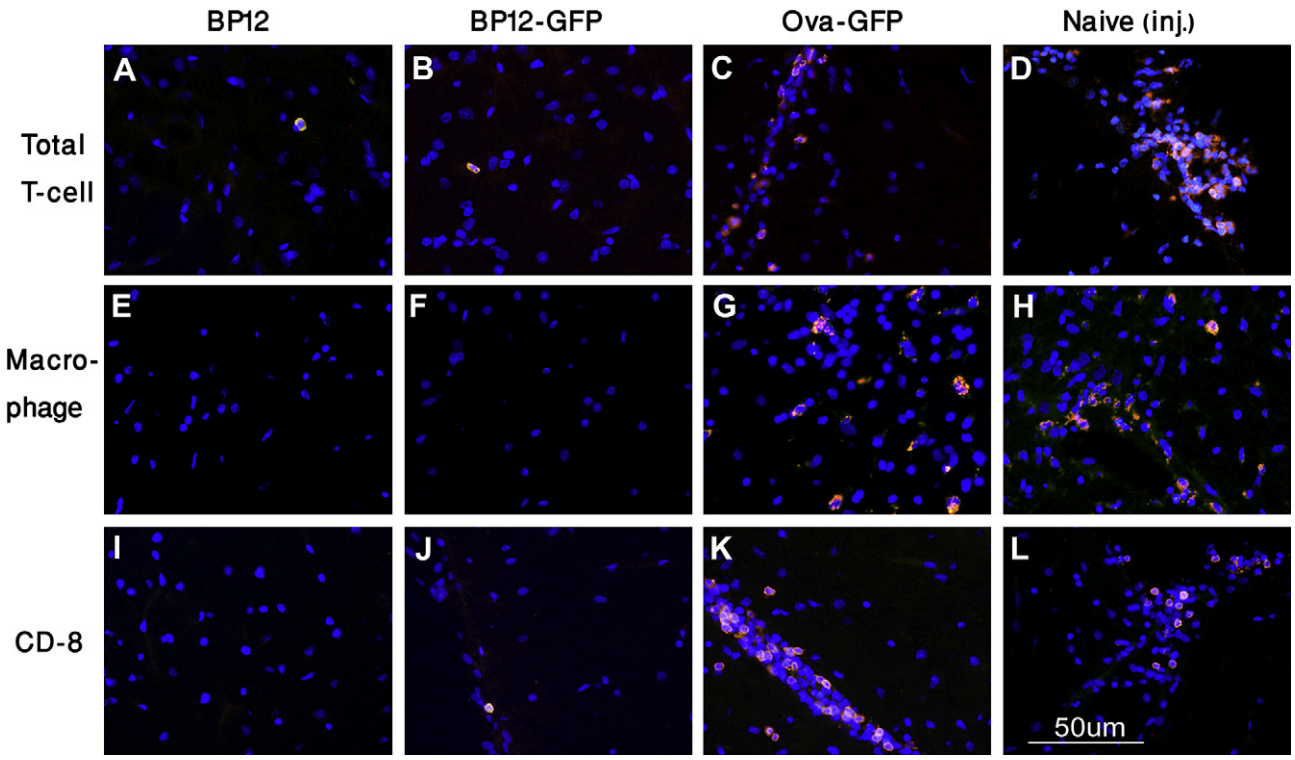

Fig. 3. Immunohistochemistry of slices from rat spinal cords: Rats were immunized three times with irradiated, activated T cells from the BP12, BP12-GFP and Ova-GFP lines or were not treated (Naïve (inj.)). All groups were injected i.v. with BP12-GFP cells. After 7 days, the spinal cords were removed and cut in a sliding microtome into 14 μm slices. Slices were stained (red) either with W3/13 staining for pan T cells (leukocyte sialoglycoprotein) or with ED-1 staining for macrophage CD68, or with OX-8 staining for $CD8^+$ cells marker. All slices were counterstained with Hoechst 33342 for cell nucleus (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Similar amounts of IFNγ were noted in the Ova-GFP and the naïve-injected groups, with 2–4% of cells found in the spinal cords secreting IFNγ at the peak of disease. In the rats protected by TCV only about 0.1% of cells could be activated to produce IFNγ, significantly less than the number of cells in the non-protected groups ($P < 0.05$ ANOVA and Tukey Kramer). IL-4 secreting cells were found almost exclusively in the rats treated by TCV with either BP12, BP12-GFP or Ova-GFP; no IL-4 production was detected in the spinal cords of the naïve-injected group. The samples were also stained for CD4 and CD8; the majority of the cells producing IFNγ or IL-4 were $CD4^+$ cells. Some $CD8^+$ cells were found amongst the cells producing low amounts of IFNγ (not shown). CNS-isolated effector cells are sensitive to PMA/ionomycin and sometimes die following stimulation with it. The GFP cells were readily observable ($FL1^{high}$ $FL2^{high}$ – not shown) in PMA non-stimulated cultures; fewer cells could be found in the same cultures stimulated with PMA/ionomycin.

Taken together, the results suggest that TCV with Ova-GFP as well as with BP12 or BP12-GFP can lead to the presence in spinal cords of cells that produce IL-4. However, only the rats vaccinated with BP12 or BP12-GFP manifested a marked decrease in cells producing IFNγ.

3.7. TCV down-regulates IFNγ secretion by spleen cells in response to MBP

IFNγ plays a pivotal role in EAE [36–38] and its expression levels correlate with the pattern and severity of the disease in the DA and Lewis rats [39]. To check whether TCV changes the amounts of IFNγ secreted in response to MBP, rat spleens were harvested and the splenocytes were used in proliferation and in ELISA assays.

Proliferation assays (over 10 assays were conducted) were not sensitive enough to detect differential responses by splenocytes between any of the groups. Similarly, intra-cellular cytokine staining of splenocytes proved inefficient – probably due to the high background levels of IFNγ expressed by irrelevant spleen cells (noise) and to the relatively low numbers of MBP-specific T cells found in the spleen (signal). However, ELISA assays for cytokine production in the media of the proliferation assays proved sensitive enough to show a consistent pattern of cytokine suppression by the assayed splenocytes.

Fig. 6 shows that the splenocytes of rats treated with Ova-GFP and those of the naïve-injected rats displayed dose-dependent IFNγ secretion in response to MBP. These responses were detected from day 4 up to day 8 following adoptive transfer of encephalitogenic T-cell lines, but could not be detected in spleens harvested on day 1 or 2 after transfer. Neither the BP12 nor the BP12-GFP TCV-treated groups showed any IFNγ secretion beyond the background secretion to medium or to Ovalbumin. In the TCV-protected groups, IFNγ secretion was similar to that of an untreated group that had not been vaccinated with T-cells or injected with BP12-GFP cells.

Secretion of IL-10 and TNF-α was also assayed. No differential IL-10 secretion was detected in none of 3 independent assays. TNF-α showed minor (2–4 pg/ml), non-significant differences between TCV-treated and non-treated groups.

The secretion of IFNγ in response to MBP recorded from day 4 to day 8 in the spleens of the TCV non-protected groups is in concurrence with the migration pattern of the anti-MBP T cells observed in Fig. 2B.

3.8. IFNγ secretion as an anti-idiotypic response to T cells used for TCV

IFNγ is secreted both by effector T cells mediating EAE and by the protective regulatory T cells induced by TCV that are specific for the TCR idiotypes of the vaccine [12,17,37]. To assess this

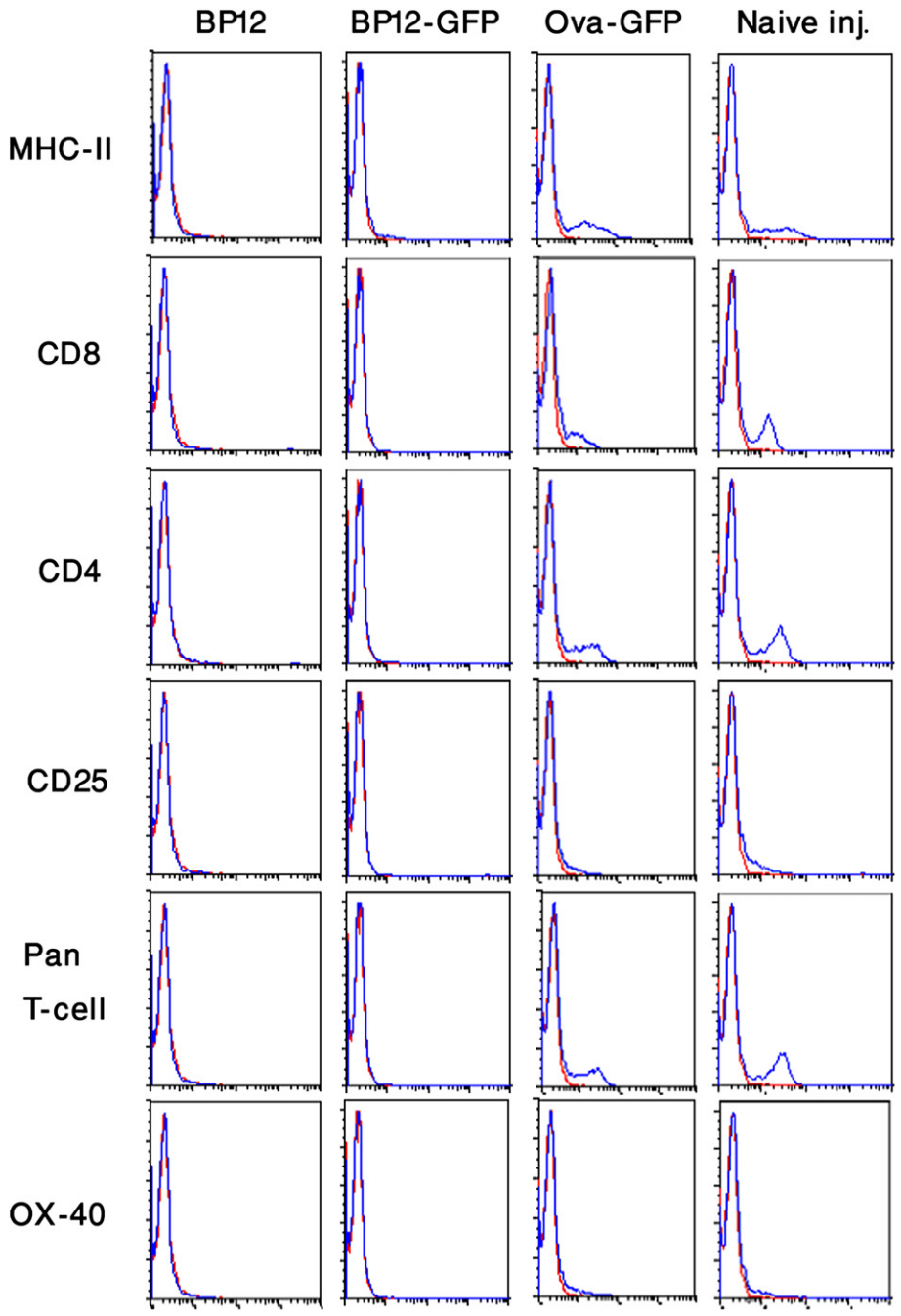

Fig. 4. TCV modulates the cellular infiltrates in the spinal cord. Female rats were vaccinated with irradiated, activated BP12, BP12-GFP or Ova-GFP T cells, or were not treated (Naïve-inj.). All groups were injected i.v. with BP12-GFP cells. Seven days later, at the peak of disease, the spinal cords were removed and cells were collected and stained with antibodies binding to MHC class II (OX-6/RT1-D), CD8 (OX-8), CD4 (W3/25), IL-2 receptor β-chain (CD25), Pan T cell (W3/13) and the OX-40 activation marker. Samples were back-gated on the size and granularity of $CD4^+$ lymphocytes. The data are representative of three or more rats per group in two similar experiments.

anti-vaccine response to the injected encephalitogenic cells, splenocytes isolated from the different groups were taken seven days after BP12-GFP cells were injected for EAE induction. The splenocytes were co-cultured with irradiated activated or irradiated-resting BP12, BP12-GFP or Ova-GFP T cells. Supernatants were analyzed by ELISA for secretion of IFNγ, IL-10 and TNF-α.

Fig. 7 shows that the secretion of IFNγ in response to activated-irradiated BP12 target cells of either of the TCV-protected groups was significantly higher than that measured in the TCV non-protected groups. IFNγ secretion to irradiated BP12-GFP targets was higher in the BP12-GFP treated group than in the Ova-GFP treated group or in the naïve-injected group. Interestingly the Ova-GFP treated group consistently responded relatively weakly to irradiated Ova-GFP T-cells. This might stem from the fact that splenocytes of all groups in these experiments were taken seven days following induction of EAE with anti-MBP T-cells that boosted the response only to anti-myelin T-cells.

Significant differential responses of cytokine secretion to the T-cell targets were found only in the IFNγ ELISA assays and were not found in the TNF-α or IL-10 ELISA assays. No significant consistent cytokine secretion was observed to irradiated-resting T cell targets in any of the experiments (not shown). Taken together,

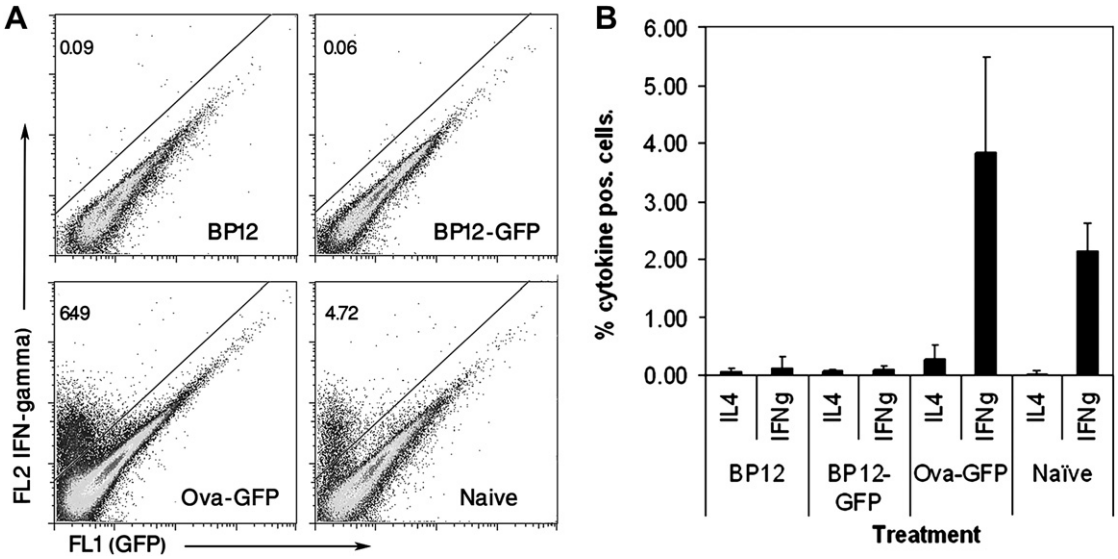

Fig. 5. Intra-cellular cytokine cytofluorometric analysis to quantify infiltrating cells producing IFNγ or IL4. Rats were treated by TCV with BP12, BP12-GFP or Ova-GFP T cells or not treated (Naive), and were then injected i.v. with BP12-GFP cells. Seven days later, at the disease peak, spinal cords were removed and cells were collected using a percoll gradient. The cells were incubated in medium containing PMA and Ionomycin. Four hours later, Golgi-plug™ was added for 3 h and the cells were fixed in 4% PFA, and stained with anti-IL4-PE, anti-IFN-γ-PE, or an isotype-control antibody-PE. (A) Representative pseudo-color dot plot of IFNγ secretion of the different groups. (B) Summary of IFNγ and IL-4 mean secretion of three rats in two identical independent experiments.

the anti-T cell IFNγ responses at the peak of the disease in the TCV-protected groups were directed to activated anti-MBP T cells only.

4. Discussion

Although much progress has been made since 1981 when TCV was first demonstrated [1], its underlying mechanisms have remained poorly understood. Recently more light has been shed on several regulatory T-cell subsets that recognize and are able to eliminate encephalitogenic T cells [4,19].

Limiting dilution assays show that TCV of MS patients is followed by an overall reduction in the numbers of T cells proliferating in response to MBP in the periphery [24]. A question that has remained unresolved is the fate of the encephalitogenic T cells in vivo following TCV: Does TCV eliminate the MBP-reactive T cells [18], or do the cells or their offspring persist with changes in their TH1/TH2 balance in the response to MBP [17], or are they driven into an inactive quiescent state, as shown following spontaneous recovery from EAE [27], or do they become anergic [26]? No TCV study as yet has directly followed the fate of the encephalitogenic effector T cells.

Here we used a tracking technique utilizing encephalitogenic T cells expressing GFP to observe and quantify the effects of TCV on these cells in vivo. We found that vaccination with a line later used to

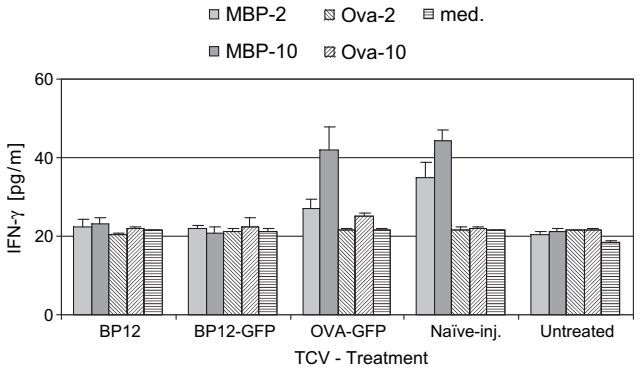

Fig. 6. ELISA assay for IFNγ secretion of splenocytes. Rats were immunized three times with irradiated activated cells from the BP12, BP12-GFP and Ova-GFP lines or were not treated (Naïve-inj.) All groups were then injected i.v. with BP12-GFP cells. After 7 days, spleens were harvested and the splenocytes were cultured with different antigens at various concentrations (2 and 10 μg/ml) for two days. Supernatants were then collected and assayed by ELISA for presence of IFNγ. Results represent the mean of triplicate wells depicted with standard error bars. Statistics: In the naïve group secretion to both concentrations of MBP is higher than secretion to medium or to Ova (ANOVA + Tukey Kramer $P < 0.01$). In the Ova-GFP group secretion to MBP-10 μg/ml is higher than the secretion to medium or to Ova (ANOVA + Tukey Kramer $P < 0.05$). The assay was repeated three times with similar results. Secretion of IFNγ following incubation with ConA was the following in pg/ml: BP12-331, BP12-GFP-231, Ova-GFP-271, Naïve (inj.)-295, Untreated-231.

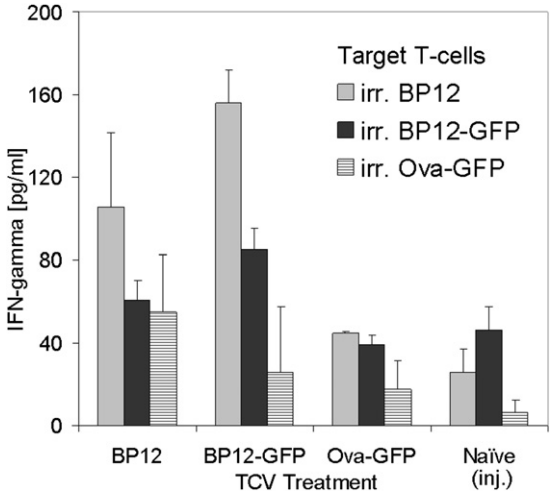

Fig. 7. TCV induces anti-idiotypic responses: Seven days following BP12-GFP injection to induce EAE, splenocytes from different groups were collected and co-cultured with various irradiated activated T-cell targets. Supernatants were collected and assayed by an ELISA for IFNγ. The background IFNγ secretion of the irradiated T cells alone was subtracted from the secretion of each group. Results represent the mean of secretion depicted with standard error bars. The target 'BP12 cells', in the BP12 and the BP12-GFP treated groups showed higher secretion than either the Ova-GFP or naïve-injected groups; for the target 'BP12-GFP cells', the BP12-GFP treated group showed higher secretion from either Ova-GFP or the naïve-injected group (ANOVA + Tukey Kramer $P < 0.05$). The assay was repeated three times with similar results.

cause the disease (BP12-GFP), or in its GFP non-expressing form (BP12) protects vaccinated rats completely from EAE mediated by the adoptive transfer of these encephalitogenic effector T cells. TCV with an activated cell line expressing GFP, but recognizing an exogenous antigen (Ovalbumin) did not protect rats from clinical EAE, and showed similar secretion of IFNγ in response to MBP in the spleen, lack of secretion of IFNγ to the encephalitogenic T cells, and exhibited large numbers GFP-labeled cells in immune organs and inside the CNS.

The small differences found between the Ova-GFP treated group and the naïve group could be associated either with an anti-ergotypic responses [10,23,44] mediated by the treatment with activated Ova-GFP cells or with a response to the foreign antigens expressed by the GFP-transduced T cells. The difference in the response to the Ova-GFP line and the BP12-GFP or BP12 lines lays in the specificity of their TCR. Under the experimental conditions we employed, anti-TCR responses were the major component apparently associated with the induction of clinically effective TCV. The expression of two foreign antigens, one of which is the immunogenic GFP protein [31] by the Ova-GFP treated group was apparently insufficient to generate a clinically relevant response against the BP12-GFP cells used to induce disease.

The GFP-expressing encephalitogenic cells were followed every 1–3 days, and differential results were observed as early as day 2 after injection. A 10- to 30-fold reduction in the numbers of GFP-expressing T cells was found in the spleens of the TCV-protected (BP12 and BP12-GFP) rats compared to the unprotected groups (Ova-GFP treated and the naïve-injected group). The numbers of GFP-expressing T cells in the spinal cords of the TCV-protected groups decreased from low numbers (10^{-4}–10^{-5}) by day 2 to almost no GFP-expressing cells (less than 10^{-5}) from day 4 onwards. Thus, anti-MBP effector cells may enter the CNS early on, but they appear to be rejected or destroyed soon thereafter. Similar results were observed in the PTLN (not shown). The PTLN is a secondary immune organ displaying the strongest infiltration of cells in the course of adoptive EAE, with up to 2% GFP-positive cells [30].

Clinically effective vaccination markedly reduced the numbers of the encephalitogenic T cells that are targeted by TCV. Nevertheless, in about half of the cases, small number of the encephalitogenic line could still be detected in the spinal cords of TCV-protected rats at the peak of disease as it occurred in control-vaccinated rats. Moreover, the GFP-labeled T cells could not be grown from splenocytes taken at the disease peak. The clinical protection by TCV can be thus correlated with massive, but not complete elimination of encephalitogenic T cells from the lymphoid organs and CNS.

Th-17 cells have been shown to be important in EAE in the mouse [40], however, only a very small number of T cells that uniquely produce IL-17 but not IFNγ can be detected in the Lewis rat EAE model we have used [38]; EAE in this model is associated primarily with IFNγ producing Th1 cells [36–38]. Functionally, we found that as early as day 4 post-injection a clear dose-dependent IFNγ response to MBP was observed in the spleens of TCV non-protected groups, while no response could be detected in the spleens of TCV-protected animals or untreated animals. Thus the functional loss of reactivity to MBP correlated with the rapid disappearance of GFP-labeled T cells in TCV-protected groups. Among many other roles, IFNγ induces major histocompatibility-complex expression in the CNS and activates microglia to become antigen-presenting and effector cells. The activation of microglial cells disturbs the immunologically privileged status of the CNS, and, subsequent to their activation, the microglia die by activation-induced cell death (ACID) [36].

Note that TCV was associated with down-regulation of the production of IFNγ by T cells responding to MBP, the target antigen for the effector T cells mediating EAE (Fig. 6). This is consistent with the ability of TCV to down-regulate clinical EAE (Fig. 1). In contrast, TCV also up-regulated the production of IFNγ by T cells responding to the activated BP12 or BP12-GFP anti-MBP effector clones used for vaccination (Fig. 7). This finding is compatible with an anti-idiotypic regulatory response induced by TCV [41]. The vaccine T cells in a resting state did not induce such a response; the activated state of the idiotypic T cells is a prerequisite for recognition by anti-idiotypic T cells [41]. IFNγ, but not IL-10 was secreted in response to irradiated BP12 or BP12-GFP cells serving as targets. Relatively low responses to the irradiated Ova-GFP targets were observed following TCV with Ova-GFP T cells (Fig. 8); this might be due to the longer duration between the time of vaccination with the irradiated Ova-GFP cells compared to the more recent injection of live encephalitogenic BP12-GFP cells that might have boosted the anti-idiotypic responses of the rats vaccinated with BP12.

Along with the reduction of encephalitogenic T cells in the spleen, the PTLN and the CNS, TCV induced a marked reduction in the inflammatory infiltrate in the CNS. The rats that had not been protected by TCV manifested high numbers of MHC class II-expressing cells, CD4 cells, and CD8 cells and notable levels of the activation markers CD25 and OX-40 in their spinal cords. These markers indicate the presence of an inflammatory process [32,39]. In contrast, none or very low numbers of inflammatory cells were found in the spinal cords of the rats protected by TCV. These results indicate that TCV not only targets the clones mediating the disease, but halts the cascade of events that leads to inflammatory infiltrates in the CNS.

Intra-cellular cytokine assays showed that the numbers of cells producing the Th_1 cytokine IFNγ was 15–30-fold lower in the TCV-protected groups compared to the controls. Small amounts of IL-4-producing cells were found exclusively in TCV-treated groups, including the rats vaccinated with the Ova-GFP T cells. IL-4 mRNA was shown to be elevated in the CNS of Lewis rats during the recovery phase of EAE [39], and in EAE-protected rats following nasal administration of MBP [42]. IL-4 was also shown to be expressed by two different types of regulatory cells participating in the induction of active tolerance to antigen in EAE [43].

TCV is being used for the treatment of a variety of autoimmune diseases including multiple sclerosis [4,5]. Several hundreds of people suffering from multiple sclerosis have been treated with TCV using vaccines composed of attenuated (irradiated) autologous, autoimmune T cells responsive to myelin antigens [44]. The results of human TCV confirm and extend the basic immunological information gathered here and in other experimental studies. Humans respond to TCV with a decrease in the number and severity of clinical attacks, and with stabilization of the lesions visualized by imaging techniques. The immune monitoring of these patients shows down-regulation of the autoimmune reactivity, up-regulation of anti-idiotypic and anti-ergotypic reactivity, and Th2 enhancement [24,45–47]. The direct in vivo effects on the encephalitogenic T cells shown here, demonstrates that T cell vaccination has significant in vivo effects on the population of encephalitogenic T cells targeted by the process of T-cell vaccination.

Conflicts of interest

The authors declare no conflicts of interest.

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