Mannan-conjugated myelin peptides prime non-pathogenic Th1 and Th17 cells and ameliorate experimental autoimmune encephalomyelitis

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Abstract

Antigen presenting cells are critical for regulating immune responses. We tested mannan-peptide conjugates for targeting myelin peptides to APC to induce T cell tolerance and resistance to experimental autoimmune encephalomyelitis (EAE). Myelin peptides conjugated to mannan in oxidized (OM) or reduced (RM) forms protected mice against EAE in prophylactic and therapeutic protocols, with OM-conjugated peptides giving best results. Protection was peptide-specific and associated with reduced antigen-specific T cell proliferation, but not alterations in Th1, Th17 or Treg cell differentiation or T cell apoptosis compared to EAE controls. OM-MOG-loaded bone marrow-derived DC showed up-regulated expression of co-stimulatory molecules, reduced PD-L1 expression and enhanced CD40-inducible IL-12 and IL-23 production, features consistent with immunogenic DC. OM-MOG induced active T cell tolerance because i.d. administration or passive transfer of OM-MOG-loaded DC suppressed ongoing EAE, while OM-MOG-vaccinated mice did not reduce the proliferation of transferred MOG-specific T cells. As *in vivo*, MOG-specific T cells cultured with OM-MOG-loaded DC showed reduced proliferation and equal Th1 and Th17 cell differentiation as those with MOG-loaded DC, but surprisingly cytokine production was unresponsive to CD40 engagement. Impaired effector T cell function was further evidenced in spinal cord sections from OM-MOG-vaccinated EAE mice, where markedly reduced numbers of $CD3^+$ T cells were present, restricted to leptomeninges and exceptional parenchymal lesions. Our results show that mannan-conjugated myelin peptides protect mice against EAE through the expansion of antigen-specific Th1 and Th17 cells with impaired proliferation responses and APC-induced co-stimulatory signals that are required for licensing them to become fully pathogenic T cells.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS that shows autoimmune features and causes demyelination, early axonal injury and progressive neurological impairment. Current treatments induce non-specific immune or T cell suppression but are only partially effective and are often associated with adverse effects. Selective treatments that target the immune cells involved in the pathological processes are needed. One approach is to identify disease-associated auto-antigens and to induce antigen-specific T cell tolerance. Several experimental strategies are effective in switching antigen-specific pro-inflammatory IFN-γ-producing Th1 cell responses to IL-4-producing Th2 cell responses or inducing regulatory T cells or anergy, including oral (Chen, Kuchroo, Inobe, Hafler, and Weiner, 1994) and inhalation (Burkhart, Liu, Anderton, Metzler, and Wraith, 1999) tolerance or altered peptide ligands (APL) which compete for Ag-specific T cell receptors and induce differential signaling through the T cell receptor (TCR) resulting in tolerance (Vergelli, Hemmer, Utz, Vogt, Kalbus, Tranquill, Conlon, Ling, Steinman, McFarland, and Martin, 1996), (Evavold and Allen, 1991). However, these approaches have not translated well into the clinic due to lack of efficacy or severe adverse effects such as hypersensitivity responses (Hafler, Kent, Pietrusewicz, Khoury, Weiner, and Fukaura, 1997), (Bielekova, Goodwin, Richert, Cortese, Kondo, Afshar, Gran, Eaton, Antel, Frank, McFarland, and Martin, 2000), (Kappos, Comi, Panitch, Oger, Antel, Conlon, and Steinman, 2000). Another approach is to harness the immune regulatory properties of professional antigen-presenting cells (APC), particularly dendritic cells (DC), which are critical not only for the induction of adaptive immune responses to foreign antigens but also for the maintenance of immune tolerance to self (Steinman, 2008).

C-type lectin receptors such as DEC205 and the mannose receptor (MR, CD206) recognize glycosylated self and nonself-antigens, are highly expressed on APC and have been used successfully for targeting peptide antigens to APC for presentation to T cells and modulating immune responses (McGreal, Miller, and Gordon, 2005). In one approach, *in vivo* targeting of antigens selectively to steady state (immature) DC by fusing them to an antibody against the DEC205 endocytosis receptor (Mahnke, Guo, Lee, Sepulveda, Swain, Nussenzweig, and Steinman, 2000) induced peripheral T cell tolerance in mice (Hawiger, Inaba, Dorsett, Guo, Mahnke, Rivera, Ravetch, Steinman, and Nussenzweig, 2001). The observation that simultaneous activation of CD40 with FGK 45 agonistic CD40 antibody changed the outcome from tolerance to prolonged T cell activation and immunity supported the concept of immature DC being involved in the induction of tolerance (Hawiger *et al.*, 2001). Mice treated with anti-DEC-205 antibody fused to myelin self-antigens myelin oligodendrocyte glycoprotein (MOG) (Hawiger, Masilamani, Bettelli, Kuchroo, and Nussenzweig, 2004) or proteolipid protein (PLP) (Stern, Keskin, Kato, Waldner, Schallenberg, Anderson, von, Kretschmer, and Strominger, 2010) peptides developed impaired T cell responses to antigen and showed resistance to EAE induction. In other approaches, peptide antigens were targeted to the MR which is expressed at high levels on APC and captures and presents soluble ligands with selectivity for heavily glycosylated proteins on the surface of yeasts, bacteria and parasites (Sallusto, Cella, Danieli, and Lanzavecchia, 1995). Peptides that are mannosylated with added sugar

units (Engering, Cella, Fluitsma, Brockhaus, Hoefsmit, Lanzavecchia, and Pieters, 1997), (Tan, Mommaas, Drijfhout, Jordens, Onderwater, Verwoerd, Mulder, van der Heiden, Scheidegger, Oomen, Ottenhoff, Tulp, Neefjes, and Koning, 1997) or chemically conjugated to the mannan polysaccharide (Apostolopoulos, Pietersz, Gordon, Martinez-Pomares, and McKenzie, 2000) show greatly enhanced presentation by major histocompatibility complex (MHC) class II and I to T cells. However, mannosylated peptides did not promote immune responses *in vivo*. Instead, immunization of mice with a mannosylated myelin autoantigen, $PLP_{139-151}$ in the presence of complete adjuvant containing *Mycobacterium tuberculosis,* showed reduced T cell proliferation responses, impaired delayed-type hypersensitivity (DTH) responses and protected mice against the induction of EAE following immunization with $PLP_{139-151}$ (Luca, Kel, van, Wouter, Koning, and Nagelkerken, 2005), (Kel, Oldenampsen, Luca, Drijfhout, Koning, and Nagelkerken, 2007), (Kel, Slutter, Drijfhout, Koning, and Nagelkerken, 2008). These findings again support the participation of APC in mediating tolerance to mannosylated self-antigens and indicate that targeting antigens to MR may be a powerful strategy to suppress autoimmune responses.

Mannan is a yeast polysaccharide that acts as a pathogen-associated molecular pattern (PAMP) and at high concentrations stimulates the activation of macrophages (Tada, Nemoto, Shimauchi, Watanabe, Mikami, Matsumoto, Ohno, Tamura, Shibata, Akashi, Miyake, Sugawara, and Takada, 2002), induces the phenotypic maturation of DC in a Toll-like receptor 4-dependent manner (Sheng, Pouniotis, Wright, Tang, Lazoura, Pietersz, and Apostolopoulos, 2006) and enhances antigen presentation and immune responses (Apostolopoulos *et al.*, 2000). Our studies have shown that conjugation of the human tumour antigen mucin 1 (MUC1) to mannan in its oxidized (OM) or reduced (RM) forms leads to its efficient presentation by MHC class I or MHC class II and the induction of T1 or T2 immune responses respectively, with OM-MUC1 giving the best IFN-γ-producing cytotoxic T cell responses and protection against tumour formation (Apostolopoulos *et al.*, 2000), (Apostolopoulos, Pietersz, Loveland, Sandrin, and McKenzie, 1995). In view of these results, in this study we investigated the potential of conjugating mannan to self-antigens as a possible strategy for diverting myelin-specific T cell responses towards an immunomodulatory profile and reducing the susceptibility of mice to EAE. We show that mannan-conjugated myelin antigens induced peptide-specific T cell tolerance and strongly ameliorated the clinical signs of EAE when administered to mice in prophylactic (vaccination) and therapeutic protocols. Surprisingly, however, tolerance was not associated with immune deviation of the effector T cell response or the induction of regulatory T cells but with the efficient induction of antigen-specific Th1 and Th17 cells that were anergic to re-stimulation with cognate antigen and showed marked reduction of encephalitogenic potential.

Materials and Methods

Mice

C57BL/6 (CD45.2), SJL/J and C57BL/6-Tg(Tcra2D2, Tcrb2D2)1Kuch/J) (2D2) mice were purchased from the Jackson Laboratory. C57BL/6 expressing EGFP under the actin promoter, TgN(act-EGFP)OsbC14-Y01-FM131, (TgEGFP) were kindly

provided by Masaru Okabe (Osaka University). CD45.1 congenic 2D2 C57BL/6 mice were kindly provided by Burkhard Becher and Melanie Greter (University of Zurich). Mice were kept under specific pathogen-free conditions in the experimental animal unit of the Hellenic Pasteur Institute. All animal procedures were performed to minimize suffering and conformed to the principles of the three Rs (replacement, refinement and reduction) following the guidelines of the EU directive for animal research 2010/63/EU. Experimentation licences were provided by the General Secretariat of Agricultural Economy and Veterinary Medicine of the Greek State according to the presidential directive 160/91. The reporting of the animal experiments in this study follows the ARRIVE guidelines.

Synthesis of myelin peptides

Peptides (murine MOG_{35-55}, $PLP_{139-151}$, $PLP_{178-191}$, myelin basic protein (MBP) $_{83-99}$) were synthesized by Fmoc/tBu methodology using the acid sensitive 2-chlorotrityl chloride (CLTR-Cl) resin (0.6-1.0 mmolCl^-/g) and N^{α}-Fmoc (9-fluorenylmethyloxycarboxyl)-protected amino acids (Tselios, Probert, Daliani, Matsoukas, Troganis, Gerothanassis, Mavromoustakos, Moore, and Matsoukas, 1999). The final products were further purified using semi-preparative reverse phase-high performance liquid chromatography (RP-HPLC). The purity of peptides was >95% as determined by analytical RP-HPLC and electron spray ionization-mass spectrometry. All peptides for conjugation with mannan were synthesized using $(Lys\text{-}Gly)_5$ at the N-terminus which acts as a linker between the peptide and mannan. The synthesis of the polypeptide $[(Lys\text{-}Gly)_5]$-(Glu, Ala, Tyr, Lys) (POL) was based on the synthesis of GA (Sela and Mozes, 2004) and involved the polymerization of five benzotriazolyl esters derived from alanine, γ-tert-butyl-glutamate, N^{ε}-butyloxycarbonyl-lysine, *O*-tert-butyl-tyrosine and N^{α}-butyloxycarbonyl-$[(N^{\varepsilon}$-butyloxycarbonyl-lysine)-glycine$]_5$. The side chain-protected units were combined in an average molar fraction 1 for $[(Lys\text{-}Gly)_5]$, 1.51 for (Glu), 4.95 for (Ala), 1 for (Tyr), 3.54 for (Lys) in a ratio of 4.95. The 1-hydroxybenzotriazole (5.85 mmol) and *N,N'*-diisopropylcarbodiimide (4.29 mmol) were added as coupling reagents in dimethylformamide solvent. The mixture was left to react for 72 h at room temperature, the solvent was removed on a rotary evaporator and the obtained oily product was precipitated from water as an amorphous pale yellow solid. The linear protected polypeptide was treated with 90% trifluoroacetic acid (TFA) in dichloromethane in the presence of 0.3% triethylsilane, anisole and H_2O as scavengers for 5 hours at room temperature. The solvents were removed on a rotary evaporator and the obtained oily product was precipitated from cold dry diethyl ether as amorphous light yellow solid. The crude peptide product was further purified by semi-preparative RP-HPLC: (column: Nucleosil C18, 5 μm, 4.6x250 mm), eluents: A, 0.08% TFA/H_2O, B, 0.08% TFA/acetonitrile, gradual gradient: from 10% to 60% B in 45 min, flow rate: 3 ml/min, detection 230 nm, 254 nm, 277 nm. All the fractions between 13-15 min were collected, lyophilized and passed through a pre-packed column Sephadex G-25 Medium to remove the low molecular weight contaminants (Mr < 1000).

Conjugation of peptides to mannan

Peptide-mannan conjugation was achieved as previously described

(Apostolopoulos, Pietersz, and McKenzie, 1996). Briefly, mannan (poly-mannose from *Saccharomyces cerevisiae;* Sigma-Aldrich Ltd) in phosphate buffer (pH 6.0) was oxidized to polyaldehydes by treating with sodium periodate. The conjugation of peptides to oxidized mannan (OM) was achieved via Schiff base formation between the free amino groups of Lys and the aldehydes of OM in bicarbonate buffer (pH 9.0). Reduction of the free aldehydes and Schiff base to alcohols and amines respectively, to form reduced mannan (RM), was achieved by treating the OM-peptide complex with sodium borohydride. Conjugates were analysed for conjugation efficiency by capillary electrophoresis as previously reported (Tselios, Lamari, Karathanasopoulou, Katsara, Apostolopoulos, Pietersz, Matsoukas, and Karamanos, 2005).

Administration of mannan-peptide conjugates to mice

In a prophylactic vaccination protocol, groups of female C57BL/6 or SJL/J mice (6-8 weeks old) were injected intradermally (i.d.) on the flanks with 100 µl PBS containing OM-MOG_{35-55} (OM-MOG), RM-MOG_{35-55} (RM-MOG), OM-$PLP_{178-191}$, OM-POL, RM-POL or an unconjugated mixture of OM and MOG_{35-55} (MOG) (in the C57BL/6 strain), and OM-$PLP_{139-151}$ or OM-MBP_{83-99} (in the SJL/J strain) (all 30 µg peptide equivalent/injection and 700 µg mannan equivalent/injection). As controls, age-matched groups of mice were vaccinated with OM or RM (700 µg), unconjugated peptide (30 µg) or PBS vehicle. Three consecutive injections were performed at 15-day intervals. Immunization for the induction of EAE was performed 15 days after the last i.d. injection. In therapeutic administration protocols, groups of female C57BL/6 mice (6-10 weeks old) were injected i.d. with 100 µl PBS containing peptide conjugates or controls, as above, at the time of immunization (day 0) and 7 later, or after the onset of clinical signs, as indicated.

EAE induction

MOG-EAE was induced in 6-8 week-old female C57BL/6 mice (17-18 week-old bone marrow chimeric mice) by subcutaneous (s.c.) tail-base injection of 30 µg of murine $MOG_{35–55}$ in 100 µl PBS emulsified in an equal volume of complete Freund's adjuvant (CFA), 15 days after the third vaccine injection. Mice received intaperitoneal (i.p.) injections of 200 ng of *Bordetella pertussis* toxin (PTx) (Sigma-Aldrich) at the time of immunization and 48 h later. In some experiments an agonistic rat IgG antibody to mouse CD40 (FGK45) (Rolink, Melchers, and Andersson, 1996) kindly provided by Antonius Rolink, University of Basel) was administered i.p. (90 µg / mouse) from day 2 post-immunization and thereafter twice-weekly for 2 weeks (total of 5 injections). PLP-EAE was induced in female SJL/J mice by s.c. tail-base injection of 150 µg of $PLP_{139-151}$ emulsified in CFA, 15 days after the third vaccine injection, without the administration of PTx. CFA used in all experiments was supplemented with 400 µg/injection of H37Ra *Mycobacterium tuberculosis* (Difco). Mice were monitored daily for the clinical signs of EAE according to the following scores: 0, normal; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or dead (0.5 gradations represent intermediate scores). Moribund animals were euthanized and given a clinical score of 5 for the remaining days of the experiment. All mice were allowed free access to food and water throughout the experiments.

TgEGFP bone marrow chimeric mice

Bone marrow cells were flushed from tibia and femur bones of naïve female TgEGFP mice and red blood cells were lysed using ammonium chloride potassium (ACK) buffer. Washed cells were transplanted i.v. to lethally irradiated female C57BL/6 recipients (5-6 weeks old) ($5x10^6$ / mouse). Efficiency of bone marrow engraftment was determined after 6 weeks of recovery by FACS analysis for GFP expression in peripheral blood cells. Mice showing >70% $EGFP^+$ blood cells were used for vaccination with OM-MOG or PBS control and induction of MOG-EAE and were sacrificed for immunohistochemical analysis of TgEGFP cell distribution in peripheral (gut, lung) and CNS tissues, and spinal cord lesion development (see below) in tissues taken at the peak of clinical disease in the control group.

Bone marrow-derived DC culture and in vivo transfer

Bone marrow-derived DC were isolated as previously described (Lutz, Kukutsch, Ogilvie, Rossner, Koch, Romani, and Schuler, 1999). Bone marrow cells were isolated from naive C57BL/6 mice as described above and cultured at 3×10^6 cells per plate in 10 ml of RPMI 1640 supplemented with 10% heat inactivated FBS for 9 days. Cells were treated with 20 ng/ml of recombinant GM-CSF (Sigma) on days 0, 3 and 6 to stimulate differentiation. Adherent cells were harvested on day 9 and loaded *in vitro* with OM-MOG, MOG, OM or PBS (10 µg/ml peptide equivalent and 233 µg/ml mannan equivalent). After 24 h, the phenotype of the $CD11c^+$ cells was evaluated by staining with fluorochrome-labeled antibodies for cell surface markers (MHC class II (anti-I-A/I-E), CD8a, CD80, CD86, CD40) and intracellular staining for PD-L1 using a FACSCalibur cytometer and CellQuest software (BD). Production of IL-23 and IL-12p70 was measured in DC supernatants by ELISA (see below). As a positive control for DC maturation, cells were stimulated with LPS (1µg/ml) derived from *E. coli* (Sigma). Cells were used in DC-T cell co-culture assays or for adoptive transfer into mice. For *in vivo* experiments, peptide-loaded DC (1×10^6 cells/mouse) were transferred i.v. to C57BL/6 recipient mice on day 8 post-immunization for MOG-EAE induction, just before the onset of clinical signs, and mice were scored daily for clinical signs of EAE.

T cell proliferation and death assays

Splenocytes or draining lymph node (DLN) cells were isolated from immunized C57BL/6 mice and cultured for 72 h in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 50 µM 2-β mercaptoethanol (Sigma), and increasing concentrations of MOG peptide. Cells were stimulated in triplicate at $2x10^6$ cells / ml in round-bottom 96-well plates (Costar). Cells were pulsed with 1 µCi / $5x10^5$ cells [^{3}H]-thymidine (Amersham Radiochemicals) for the last 16 h of culture. [^{3}H]-Thymidine incorporation was measured by liquid scintillation counting (Wallac). Results are expressed as the stimulation index (SI) calculated from the radioactivity counts per minute (cpm) of cells cultured in the presence of peptide divided by cpm of cells cultured in medium alone.

To measure T cell proliferation *in vivo*, splenocytes and DLN cells were isolated from CD45.1 congenic MOG_{35-55}-specific T cell receptor (2D2) transgenic donor mice (Bettelli, Pagany, Weiner, Linington, Sobel, and Kuchroo, 2003) and

labeled with 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE) (CFDA-SE; Molecular Probes). Cells were washed and resuspended at a concentration of 10^7/ml in PBS. CFSE was added at a final concentration of 5 µM and incubated for 5 min at RT. The reaction was stopped by washing the cells with RPMI 1640 (Life Technologies) containing 10% FCS. Cells (10 x 10^6 cells/mouse) were injected i.v. in the tail vain of vaccinated, CD45.2 recipient mice on day 2 post-immunization for MOG-EAE induction. On day 7 of EAE DLN cells were isolated and analyzed by flow cytometry.

Measurement of $CD4^+$ T cells undergoing apoptotic cell death after prolonged stimulation with peptide-loaded DC in DC-T cell co-cultures (assayed at days 6, 7, 9, 12 of culture) was made using the FITC Annexin V apoptosis detection kit (BD Pharmingen).

Cell phenotyping and cytokine production

For intracellular cytokine staining mononuclear cells stimulated for 3h with PMA and ionomycin in the presence of Brefeldin A, were fixed in 2% paraformaldehyde solution in PBS for 10 min at room temperature and permeabilized with 0.5% wt/vol saponin prior to staining for surface markers and intracellular cytokines using fluorochrome-labeled antibodies (anti-CD4, clone L3T4; anti-CD45.1, clone A20; anti-CD11c, clone HL3; anti-CD8a, clone 53-6.7; anti-CD40, clone 3/23; anti-CD80, clone 16-10A1; anti-CD86, clone GL1; anti-I-A/I-E (MHC class II), clone 2G9; anti-IL-17, clone TC11-18H10; anti-IFN-γ, clone XMG1.2; anti-FoxP3, clone; FJK-16s; anti-PD-L1 (CD274), clone MIH5; IgG1 isotype control, clone R3-34; IgG2a isotype control, clone B39-4 all from BD Biosciences). Data was acquired and analyzed with a FACSCalibur cytometer and CellQuest software (BD) and with FlowJo, version 10.0.6 (Tree Star). The production of IL-23 and IL-12p70 by DC was measured in cell supernatants by mouse ELISA Ready-SET-Go kits (e-Bioscience) (sensitivity for IL-12p70 at 15 pg/ml and for IL-23 at 8 pg/ml).

DC-T cell antigen presentation assays

Splenocytes and DLN cells were isolated from 2D2 mice and co-cultured at 2 x 10^5 cells/well in 96 well plates with peptide-loaded DC at different ratios. All combinations of responder and stimulator cells were cultured in triplicate for 72 h. Cell proliferation was measured by [^{3}H]-thymidine incorporation and cytokine production was measured by intracellular cytokine staining as described above. In experiments with CD40 costimulation, cells were incubated for 72 h in the absence or presence of agonistic anti-CD40 antibody FGK45 (10 µg/ml).

For T helper cell polarization experiments, splenocytes and peripheral LN cells were isolated from 2D2 mice and co-cultured with peptide-loaded, *in vitro* matured, bone marrow-derived DC. The polarization conditions were: Th1 culture; IL-12 (10 ng/ml, Peprotech), anti-IL4 (10 µg/ml, R&D systems): Th17 culture; IL-6 (50 ng/ml, Peprotech), IL-23 (10 ng/ml, eBioscience), TGF-β (5 ng/ml, Peprotech), anti-IFN-γ (10 µg/ml, R&D systems): Treg culture; TGF-β (10 ng/ml, Peprotech), anti-IFN-γ (10 µg/ml, R&D systems), anti-IL-4 (10 µg/ml, R&D systems). On day 3 of culture, Th1 and Treg polarizing cultures were supplemented with IL-2 (10 ng/ml, Peprotech) and Th17 polarizing cultures were supplemented with IL-23 (10 ng/ml). Cells were harvested on day 5 for FACS analysis of lineage markers.

Histopathological analysis

Mice were transcardially perfused with ice-cold 4% paraformaldehyde in PBS under deep anaesthesia. CNS tissues were post-fixed in the same fixative for 3 h at 4°C and processed for standard histopathological analysis. Inflammation was visualized by staining with H&E and demyelination was demonstrated by Luxol Fast Blue/periodic acid-Schiff staining. Quantification of inflammation and demyelination was done in a blinded manner. Inflammation in the spinal cord was determined by absolute true quantification; the numbers representing inflammatory infiltrates/mm^2 of tissue. In the brain a semi-quantitative scoring of inflammation was used in which, 0.5 means single perivascular infiltrates and 1 means multiple inflammatory infiltrates. Demyelination was also evaluated by semi-quantitative scoring as follows: 0.5: single perivascular sleeves of demyelination, 1: ubiquitous perivascular or subpial demyelination, 2: confluent demyelinated plaques, 3: profound focal demyelination, involving about 1/2 of the spinal cord white matter at least in one spinal cord segment, 4: extensive demyelination, for instance complete demyelination of spinal cord white matter at least in one segment of the spinal cord.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections (4 µm) to evaluate tissue distribution of TgEGFP bone marrow-derived immune cells, $CD3^+$ T cells as well as production of the p22phox subunit of NADPH oxidase and iNOS by inflammatory macrophages. Antigen retrieval in paraffin sections was performed in a food steamer in citrate buffer (pH 6) for 40 min. The primary Abs used were polyclonal rabbit anti-GFP IgG (1/200; Molecular Probes; A11122), monoclonal rabbit anti-CD3 (1/2000; Neomarkers; RM-9107), polyclonal rabbit anti-p22phox (1/100; Santa Cruz Biotech; sc-20781) and polyclonal rabbit anti-rat iNOS (1/375; Chemicon; AB1631) followed by biotinylated secondary anti-IgG Ab (1/500; Vector laboratories). An avidin-biotin complex was used for detection of the biotinylated Abs and immune complexes were visualized by incubation with 3,3'-diaminobenzidine tetrachloride (DAB) (both from Vector laboratories).

Statistical analysis

All statistical analyses were performed with Sigma Stat 3.5, Sigma Plot 11 and Microsoft Excel. All data are given as mean ± standard error of the mean (SEM). Student's t test and Kruskal-Wallis test were used. Results were considered statistically significant when $p<0.05$.

Results

Administration of OM- or RM-MOG prophylactically or therapeutically protects mice against MOG-EAE

To examine the effect of the H-2^b binding MOG_{35-55} peptide (MOG) conjugated to oxidized mannan (OM-MOG) or reduced mannan (RM-MOG) upon the development of MOG-EAE, we delivered peptide conjugates to C57BL/6 (H-2^b) mice in

a prophylactic vaccine protocol prior to the induction of EAE by immunization with MOG emulsified in CFA and administration of *Bordetella pertussis* toxin (MOG/CFA/PTx). Three intradermal (i.d.) injections of OM- or RM-MOG, but not unconjugated OM, RM, MOG or a mixture of unconjugated OM and MOG (OM/MOG) in dilute soluble form and spaced at 15 day intervals prior to immunization with MOG/CFA/PTx, protected mice from the subsequent development of EAE compared to PBS (Fig. 1a, Supplementary Fig. 1a), with OM-MOG giving increased protection when compared to RM-MOG (Fig. 1a).

We next tested the effects of mannan-conjugated peptides when administered in therapeutic protocols in the presence of adjuvants and PTx. In one approach peptide conjugates were injected i.d. on the day of EAE induction with MOG/CFA/PTx and again seven days later. OM-MOG and RM-MOG, but not unconjugated OM, RM, or MOG, showed protective effects compared to PBS. With this protocol protection by OM-MOG and RM-MOG showed statistically similar levels of protection up to the last time point tested (Fig. 1b). In a second approach we injected OM-MOG after the onset of MOG-EAE, when mice have reached at least clinical score 2 and found that OM-MOG rapidly reduced the severity of ongoing disease, with the clinical condition of the experimental animals progressively improving upon each injection (Fig. 1c).

In conclusion, i.d. administration of OM-MOG strongly protected mice against clinical MOG-EAE in all prophylactic and therapeutic treatment protocols tested, even in the presence of PTx and activated encephalitogenic T cells, and OM-conjugated peptides were chosen for subsequent experiments investigating the mechanism of tolerance induced by mannan-conjugated self-antigens.

Vaccination with OM-MOG and RM-MOG protects mice against EAE neuropathology

To determine whether amelioration of the clinical signs of MOG-EAE by OM- and RM-MOG was associated by less severe neuropathology, vaccinated mice were sacrificed 24 days after EAE induction for neuropathological analysis of spinal cord and brain tissues (Fig. 1d and e). PBS-treated mice showed substantial mononuclear cell infiltration and extensive demyelination in the spinal cord. In contrast, mice vaccinated with OM-MOG showed reduced inflammatory cell infiltration and little or no demyelination in the spinal cord. RM-MOG-vaccinated mice showed reduced neuropathology compared to PBS-treated mice. Mice vaccinated with unconjugated MOG, OM or RM showed equivalent spinal cord pathology to PBS-treated animals (Fig. 1d). A quantitative assessment of demyelination and inflammation in each individual mouse confirmed that CNS pathology in OM-MOG-vaccinated mice was significantly reduced compared to PBS-treated mice (Fig. 1e).

We conclude that the administration OM-MOG as a prophylactic vaccine in mice inhibits the accumulation and infiltration of immune cells into the CNS parenchyma during EAE and the development of inflammatory and demyelinating lesions.

Protection from EAE by mannan-conjugated peptides is peptide-specific

To determine whether protection by mannan-peptide conjugates can apply to other CNS antigens and also whether it depends on peptide specificity, we next performed peptide criss cross experiments where we complemented the MOG-EAE

model with another EAE model in SJL/J mice (H-2^s) in which disease is induced by immunization with the H-2^s binding peptide, proteolipid protein 139-151 (PLP) (McRae, Kennedy, Tan, Dal Canto, Picha, and Miller, 1992). OM was conjugated to PLP (OM-PLP) or another H-2^S binding peptide, myelin basic protein 83-99, which itself is capable of inducing EAE when used to immunize SJL/J mice (Miller, Karpus, and Davidson, 2010), as peptide control. Groups of SJL/J mice were vaccinated, as above, with OM-$PLP_{139-151}$, OM-MBP_{83-99} or PBS. Fifteen days after the last injection, EAE was induced by immunization with $PLP_{139-151}$ in CFA (PLP/CFA). Control mice vaccinated with PBS developed acute severe clinical signs typical of PLP-EAE (Fig. 2a). As in the MOG-EAE model, mice vaccinated with mannan-conjugated to cognate peptide, OM-$PLP_{139-151}$, but not irrelevant peptide OM-MBP_{83-99}, were strongly protected against PLP-EAE (Fig. 2a). In the crossover experiment using the MOG-EAE model, OM was conjugated to another H-2^b-binding peptide, PLP 178-191 which itself is capable of inducing EAE in C57BL/6 mice (Tompkins, Padilla, Dal Canto, Ting, Van, and Miller, 2002), as peptide control. Groups of C57BL/6 mice were vaccinated with OM-MOG_{35-55}, OM-$PLP_{178-191}$ or PBS and fifteen days after the last injection EAE was induced by immunization with MOG/CFA/PTx. As predicted, mice vaccinated with OM-MOG_{35-55}, but not OM-$PLP_{178-191}$, were strongly protected against MOG-EAE (Fig. 2b).

In a second approach we used the MOG-EAE model to test the prophylactic efficacy of OM and RM conjugated to a polypeptide mixture of synthesized randomly from four amino acids (L-Glutamic acid, L-Lysine, L-Alanine and L-Tyrosine) based on GA [30] (Glu, Ala, Tyr, Lys) (POL), which has been shown to induce T cell tolerance in MOG-EAE and is used in the treatment of MS (Sela *et al.*, 2004). Groups of C57BL/6 mice were vaccinated, as above, with OM- and RM-POL conjugates or unconjugated OM, RM or POL with or without the [$(Lys\text{-}Gly)_5$] linker. Fifteen days after the last injection, mice were immunized with MOG/CFA/PTx to induce EAE. Under these conditions all mice, including those injected with POL, developed EAE with equivalent severity and timing as PBS-injected controls (Supplementary Fig. 1b).

We conclude that the induction of T cell tolerance by mannan-conjugated peptides applies in different EAE models and is peptide-specific, at least during the time frame of our experiments, applying only to the peptide used to immunize mice for the induction of EAE.

Antigen-specific responses of T cells exposed to OM-MOG show reduced proliferation but efficient expansion of Th1, Th17 and Treg populations

To determine whether the therapeutic effects of OM-MOG in MOG-EAE were associated with alteration of antigen-specific immune responses, we compared T cell responses to MOG immunization in mice that had been previously treated with the different vaccination components. Mice were immunized with MOG/CFA/PTx 15 days after the last vaccine injection and DLN and/or splenocytes were isolated at different time points prior to and after the onset of clinical signs in the PBS-treated group, re-stimulated with MOG peptide *in vitro* and analyzed for proliferation and effector T cell cytokine production. Time points analyzed were pre-onset (days 7 & 10 post-immunization), peak of EAE (day 15) and chronic EAE (day 25). Antigen-specific proliferation responses were measured in splenocytes isolated 25 days post-immunization with MOG/CFA/PTx and restimulated with MOG_{35-55} peptide *in vitro*.

Lymphocytes from mice vaccinated with OM-MOG or RM-MOG, but not OM, RM, or MOG showed reduced antigen-induced proliferation responses compared to PBS (Fig. 3a, and data not shown). Similar results were obtained using DLN cells isolated from vaccinated mice 10 days post-immunization with MOG/CFA and restimulated with MOG_{35-55} peptide *in vitro* (data not shown). The percentages of total $CD4^+$ T cells isolated from the DLN and spleens of mice from different vaccination groups were similar following immunization (Supplementary Fig. 2).

We next examined whether T cells isolated from naïve MOG_{35-55}-specific T cell receptor (2D2) transgenic donor mice (Bettelli *et al.*, 2003), and transferred into recipient mice that had been previously vaccinated with OM-MOG, would acquire tolerance at a time when OM-MOG was no longer present in the recipient mouse. Splenocytes were isolated from 2D2 CD45.1 donor mice, labelled *ex vivo* with CFSE and adoptively transferred into vaccinated CD45.2 recipient mice, 17 days after the last i.d. injection of OM-MOG and 2 days after immunization with MOG/CFA/PTx. Analysis of fluorescence in the transferred CFSE-labeled 2D2 CD45.1 T cells isolated from DLN 7 days post-immunization showed that, unlike the endogenous cells, there were no differences in the proliferation of exogenously administered cells between mice vaccinated with OM-MOG, OM, MOG or PBS (Fig. 3b). This result indicates that the presentation of OM-MOG to T cells is required for the induction of long-lasting reduction in antigen-specific T cell proliferation capacity and that tolerance is not transferred in the absence of OM-MOG.

Unexpectedly, the expansion of effector T helper cell populations was unaffected in OM-MOG-vaccinated mice, as Th1 and Th17 populations were equal to those in control mice. Thus, $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17^+$ DLN (day 7) (Fig. 4c) and $CD4^+IL\text{-}17^+$ DLN (Fig. 4d, left panel) and splenocytes (Fig. 4d, right panel) (days 10 and 15) showed no differences from control mice or other vaccination groups as measured by intracellular cytokine staining. Also, no IL-4 was detectable upon restimulation of cells from any of the vaccinated groups (data not shown), indicating that there was no overt emergence of Th2 cells in OM-MOG-vaccinated mice. In one experiment, in which DLN were isolated on day 13 post-immunization with MOG/CFA/PTx from mice that were treated with OM-MOG or OM on days 0 and 7 post-immunization (therapeutic protocol), the production of IL-10 in CD4+ T cells was reduced in OM-MOG-treated mice compared to OM-treated controls (Fig. 3e).

The induction of regulatory T cells is a critical mechanism of tolerance in EAE where they limit encephalitogenic T cell expansion and function (Kohm, Carpentier, Anger, and Miller, 2002). We measured the induction of regulatory T cell populations in mice that were vaccinated with the various peptide conjugates and subsequently immunized with MOG/CFA. DLN were isolated 7 days post-immunization and $CD4^+$ T cells were analyzed for Foxp3 expression, the signature transcription factor of regulatory T cells. The proportions of $CD4^+$ $Foxp3^+$ T cells were not significantly altered in OM-MOG-vaccinated mice compared to PBS-, OM- and MOG-vaccinated control groups (Fig. 3f).

In conclusion, OM-MOG exposure of T cells induces lasting peripheral T cell tolerance that is associated with reduced antigen-specific proliferation responses but not altered expansion of effector Th1 and Th17 T cells, immune deviation or the induction of regulatory T cell populations.

OM-MOG-loaded DC up-regulate maturation markers, Th1 and Th17 polarising cytokines, down-regulate PD-L1 and are sufficient to transfer tolerance into EAE mice

The MR is expressed by APC, mainly macrophages and DC (Sallusto *et al.*, 1995), (Linehan, Martinez-Pomares, Stahl, and Gordon, 1999) and mediates innate activation signals from PAMPs such as mannan (Tada *et al.*, 2002). To investigate whether murine DC can present OM-MOG to T cells and participate in the induction of tolerance in vaccinated mice we cultured bone marrow-derived DC, loaded them with the vaccine components *ex vivo* and used them for antigen presentation assays *in vitro* and adoptive transfer experiments in mice. Peptide-loaded DC were first analysed for the expression of DC maturation markers. $CD11c^+CD8^-$-gated DC, loaded with unconjugated OM or OM-MOG (OM-MOG DC), showed increased expression CD40, CD80 and CD86 compared to those loaded with MOG and PBS, showing levels of CD40 and CD80 at least as high as in LPS-treated DC (1 µg/ml), while proportions of MHC class II-expressing cells were equivalent in the peptide-loaded and control populations (Fig. 4a). These results confirm that OM, in either unconjugated or peptide-conjugated form, induces phenotypic maturation of *in vitro* DC. In contrast, $CD11c^+CD8^-$-gated DC loaded with MOG (MOG DC) did not show upregulation of activation markers compared to control PBS DC confirming that unconjugated peptide does not induce phenotypic maturation of DC (Fig. 4a).

We next compared effector cytokine secretion by OM-MOG and MOG DC in the absence or presence of CD40 costimulation by FGK 45 agonistic anti-CD40 antibody (Rolink *et al.*, 1996). CD40 is a costimulatory receptor on APC that is triggered by CD40 ligand produced by strongly activated $CD4^+$ T cells. It boosts immune responses through the induction of T cell polarizing cytokines and is essential for licensing DC to induce development of functional effector cells (Albert, Jegathesan, and Darnell, 2001), (Fujii, Liu, Smith, Bonito, and Steinman, 2004), (Sporri and Reis e Sousa, 2005), (Iezzi, Sonderegger, Ampenberger, Schmitz, Marsland, and Kopf, 2009) and for the development of MOG-EAE (Becher, Durell, Miga, Hickey, and Noelle, 2001), (Iezzi *et al.*, 2009). OM-MOG DC showed increased production of the Th1 and Th17 polarising cytokines, IL-12 and IL-23 respectively, compared to MOG DC and production in both cultures was increased by CD40 engagement with FGK 45 (Fig. 4b). We next tested whether OM-MOG-induced tolerance could be associated with altered production of programmed death ligand-1 (PD-L1), an APC-expressed ligand that negatively regulates TCR signalling via PD-1 on T cells and actively terminates antigen-specific T cell responses including those that induce EAE (Carter, Leach, Azoitei, Cui, Pelker, Jussif, Benoit, Ireland, Luxenberg, Askew, Milarski, Groves, Brown, Carito, Percival, Carreno, Collins, and Marusic, 2007). Interestingly, we found production of PD-L1 to be decreased in $CD11c^+$ OM-MOG DC compared to MOG-, OM- and PBS-loaded DC (Fig. 4e).

To directly evaluate whether *in vitro* grown, OM-MOG-loaded DC showing expression of maturation markers and up-regulated Th1 and Th17 polarising cytokines, are sufficient to actively induce tolerance in mice with on-going EAE, we adoptively transferred MOG-, OM-MOG-, OM- or PBS-loaded DC into non-vaccinated recipient mice, 8 days after EAE induction by immunization with MOG/CFA/Ptx. Previous studies have shown that bone marrow-derived DC from Lewis rats with EAE (Xiao, Huang, Yang, Xu, and Link, 2001) or $CD11c^+CD11b^+$ DC isolated from MOG-

tolerized mice (Li, Zhang, Chen, Xu, Fitzgerald, Zhao, and Rostami, 2008) are sufficient to transfer peptide-specific resistance to EAE in naïve recipients, a property associated with their immature status. While none of the transferred DC populations prevented disease onset, both MOG DC and OM-MOG DC, and not OM DC or PBS DC, reduced the severity of clinical symptoms during the chronic phase of disease compared to PBS DC (Fig. 4c). Together these results show that immature MOG DC and phenotypically mature OM-MOG DC are both competent and sufficient to actively transfer tolerance and reduce clinical symptoms in mice with on-going EAE, although possibly by different mechanisms.

OM-MOG DC induce normal maturation of MOG-specific Th1 and Th17 responses but reduced T cell proliferation and CD40 costimulation responses

To further assess the ability of OM-MOG DC to present cognate antigen to T cells and to investigate the cellular mechanism of OM-MOG-induced tolerance we performed *in vitro* antigen presentation assays between peptide-loaded DC and MOG-specific 2D2 T cells in the absence of presence of FGK 45 , and measured T cell responses as readout. Bone marrow-derived DC were loaded with peptide conjugates *ex vivo*, co-cultured with lymphocytes from 2D2 transgenic mice under neutral, and in some experiments Th1, Th17 and Treg polarising conditions and T cell proliferation and cytokine responses to MOG were measured. Both MOG and OM-MOG DC induced robust proliferation responses in 2D2 T cells and these responses were further enhanced by the presence of FGK 45. However, proliferation induced by OM-MOG DC was significantly lower than that induced by MOG-loaded DC at two DC-T cell ratios tested, 1:1 (data not shown) and 1:5 (Fig. 5a). MOG and OM-MOG DC also efficiently and equally induced the maturation of Th1 and Th17 effector T cell populations under neutral (Fig. 5b), as well as Th1, Th17 and Treg polarizing (Supplementary Fig. 3), conditions, an effect in line with the observation of normal priming of $CD4^+IL\text{-}17^+$ and $CD4^+IFN\gamma^+$ T cells in response to MOG immunization in OM-MOG-vaccinated mice (see Fig. 3). Interestingly however, while FGK 45 boosted cytokine production induced by MOG DC, it had no effect on IFNγ or IL-17 production induced by OM-MOG DC (Fig. 5b) suggesting that OM-MOG-stimulated T cells, besides showing reduced antigen-specific proliferation, also show anergy to CD40 co-stimulation of effector cytokine production.

To investigate whether the reduced proliferation of MOG-specific $CD4^+$ T cells observed *in vitro* and *in vivo* was associated with increased T cell death, we assessed cell surface annexin V staining in the DC-T cell co-cultures after prolonged stimulation of 2D2 T cells with peptide-loaded DC. The translocation of annexin V to the cell surface of $CD4^+$ T cells is an early event in apoptotic death occurring after cell activation. $CD4^+$ T cell surface annexin V staining was not increased in OM-MOG or MOG DC cultures compared to PBS and OM DC cultures when measured at several culture time points (representative culture at day 7 postactivation; Fig. 5c). The finding that OM-MOG DC do not increase apoptosis in MOG-specific T cells indicates that the reduced proliferation of MOG-specific lymphoblasts seen in OM-MOG-vaccinated mice and OM-MOG DC-T cell co-cultures is not due to increased cell death.

Since cell types other than DC express MR, including macrophages, some endothelial cells and perivascular microglia (Linehan *et al.*, 1999), we next

investigated whether CD40 co-stimulation of APC *in vivo* could change the outcome of EAE resistance in OM-MOG-vaccinated mice. We administered FGK45 (90 µg/ injection) i.p. twice-weekly starting 17 days after the last i.d. injection of OM-MOG and 2 days after immunization with MOG/CFA/PTx for the induction of EAE. FGK45 administration caused a temporary delay but subsequent enhancement of clinical signs in control PBS-vaccinated mice compared to non-treated controls (Fig. 5d). In contrast, FGK45 did not alter the susceptibility of OM-MOG vaccinated mice to disease. Both FGK45-treated and non-treated OM-MOG-vaccinated mice remained disease-free up to the last time-point studied (Fig. 5d).

To investigate whether T cells exposed to OM-MOG DC showed anergy we repeated the proliferation assay in the presence of exogenous IL-2. The proliferation of 2D2 T cells stimulated with OM-MOG DC was significantly increased by IL-2, up to the level shown by control cells cultured with MOG DC. The proliferation of 2D2 T cells stimulated with MOG DC was not further stimulated by IL-2 (Fig. 5e)

We conclude that OM-MOG DC efficiently provide the initial activation signals for antigen-specific Th1 and Th17 T cell responses but that these T cells show reduced proliferation responses to MOG and effector cytokine production is not boosted in response to CD40 co-stimulation. This, together with the additional finding that exogenous IL-2 reversed the unresponsiveness of T cells to antigen stimulation shows that OM-MOG induces differential TCR signaling in MOG-specific T cells that results in selective functional anergy.

$CD3^+$ T cells infiltrate spinal cord leptomeninges and activate inflammatory macrophages in small lesions but do not induce clinical signs in OM-MOG-vaccinated mice

To understand why the antigen-specific Th1 and Th17 cells that are primed in OM-MOG-vaccinated mice are not able to initiate EAE we next investigated whether T cells are able to infiltrate the parenchymal tissue of the spinal cord and trigger downstream effector mechanisms such as macrophage recruitment and activation. First we attempted to isolate mononuclear cells from the spinal cords of OM-MOG- and PBS-vaccinated EAE mice at the peak of disease in the PBS group (with clinical scores 3.5-4) and to measure the proportions of $CD4^+$ T cells. In PBS-vaccinated mice CNS-infiltrating mononuclear cells were readily isolated and contained approximately 15% $CD4^+$ T lymphocytes as measured by flow cytometry (data not shown). However, only very low numbers of CNS-infiltrating cells could be recovered from OM-MOG-vaccinated mice and flow cytometry analysis of these cells was not possible.

We next investigated the tissue distribution of activated MOG-specific T cells and the extent of immune cell infiltration into the spinal cord during EAE in OM-MOG-vaccinated mice by generating bone marrow chimeric mice. We reconstituted lethally-irradiated C57BL/6 wild-type mice with bone marrow cells isolated from double transgenic TgEGFP x 2D2 mice. Chimeric mice showing ≥70% reconstitution of blood leukocytes by GFP^+ cells, as measured by flow cytometry, were vaccinated and immunized for MOG-EAE. Immunohistochemical analysis of gut, lung and spinal cord taken at the peak of EAE in the PBS-treated group using anti-GFP antibodies showed bone marrow-derived cells distributed in the lamina propria and submucosa of the gut, Peyer's patches of the ileum, lung tissue and large confluent lesions in the white

matter of the spinal cord (Supplementary Fig. 4). Immunostaining of serial sections with anti-CD3 antibody did not reveal any T cells in gut or lung but showed numerous T cells distributed throughout the spinal cord lesions (Fig. 6a). OM-MOG-vaccinated mice showed a similar distribution as controls of GFP-immunoreactive cells in the gut and lung and no $CD3^+$ T cells were detected in these tissues. In the spinal cord, consistent with the histopathological analysis (Fig. 1d), the numbers of recruited GFP-immunoreactive (Supplementary Fig. 4) and CD3-immunoreactive cells (Fig. 6a) were markedly reduced compared to EAE controls. Both $CD3^+$ T cells and GFP-immunoreactive immune cell infiltrates were restricted to the leptomeninges or occasional small compact lesions in the white matter (Fig. 6a). Interestingly, these lesions were detected in mice that displayed no clinical signs on any day of follow-up.

To address whether the reduced antigen-specific T cell proliferation measured in secondary lymphoid organs of OM-MOG-vaccinated mice following MOG immunization, and in 2D2 T cells co-cultured with OM-MOG DC, might be responsible for reduced immune cell infiltration of the spinal cord in OM-MOG-vaccinated mice during EAE we performed double immunofluorescence staining using antibodies to CD3 and Ki67, a marker of cell proliferation. In PBS-treated mice, a small proportion of $CD3^+$ T cells distributed throughout the tissue lesions and leptomeninges showed Ki67-immunoreactivity (Fig. 6b), indicating that proliferation of T cells occurs in white matter lesions during EAE. This is consistent with the findings of Wekerle and colleagues that T cells enter the brain in the "migratory" phenotype, encounter their specific antigen at APC in meninges and perivascular spaces, proliferate and acquire the ability to pass the astrocytic glia limitans and invade the CNS parenchyma (Lodygin, Odoardi, Schlager, Korner, Kitz, Nosov, van den Brandt, Reichardt, Haberl, and Flugel, 2013), (Mues, Bartholomaus, Thestrup, Griesbeck, Wekerle, Kawakami, and Krishnamoorthy, 2013). In OM-MOG-treated mice, similarly small proportions of $CD3^+$ T cells showed Ki67-immunoreactivity (Fig. 6b) although, as mentioned above, the overall numbers of T cells were markedly reduced compared to EAE controls, and were differentially distributed, being detected only in leptomeninges (Fig. 6a). No double-labeled CD3+Ki67+ cells were detected in the parenchymal lesions in OM-MOG-vaccinated mice.

We further investigated whether T cells in OM-MOG-vaccinated mice can trigger downstream effector mechanisms such as macrophage recruitment and activation in the spinal cord, by analyzing the accumulation of p22phox, which is an essential subunit of NADPH oxidases, and iNOS, in sections from mice that had been vaccinated with OM-MOG or PBS, as shown in Fig. 1d. In the active MOG-EAE model used in this study, tissue injury is associated with massive infiltration of the tissue by $CD3^+$ T cells and the presence of numerous macrophage-like cells showing p22phox and iNOS expression (Schuh, Wimmer, Hametner, Haider, Van Dam, Liblau, Smith, Probert, Binder, Bauer, Bradl, Mahad, and Lassmann, 2014). Here, PBS-vaccinated mice showed typical spinal cord lesions of MOG-EAE, with numerous $CD3^+$ T cells and p22phox- and iNOS-immunoreactive macrophage-like cells (Fig. 6b, first and second columns). Surprisingly, OM-MOG-vaccinated mice also showed sparse infiltration of the spinal cord by $CD3^+$ T cells and p22phox- and iNOS-immunoreactive macrophage-like cells which were much fewer in number and, as mentioned above, limited to

leptomeninges or to exceptional compact white matter lesions (Fig. 6b, third and fourth columns).

Overall, these data show that antigen-specific $CD3^+$ T cells traffic to the spinal cord in OM-MOG-vaccinated mice during the development of EAE, and locally activate macrophage-like cells to produce reactive oxygen and nitrogen species, although numbers are greatly reduced and mainly restricted to leptomeninges compared to the large confluent white matter infiltrates typical of EAE in PBS-treated control mice.

Discussion

In this study we describe a method for targeting myelin peptide antigens to APC and inducing robust peptide-specific T cell tolerance in mice, protecting them against the development of EAE when administered as i.d. injection in prophylactic (vaccination) or therapeutic protocols and in the presence of strong immune stimulants such as PTx and agonistic anti-CD40 antibody. EAE-inducing myelin peptide epitopes were synthesized with a $(Lys\text{-}Gly)^5$ linker and chemically conjugated to the polysaccharide mannan, a ligand for the MR, in its oxidized or reduced form. Specifically, we conjugated mannan to H-2^b binding ($MOG_{35\text{-}55}$) and H-2^s binding ($PLP_{139\text{-}151}$) myelin peptides and show that they protect mice against the induction of EAE in two different models, a chronic form induced in C57BL/6J (H-2^b) mice by immunization with MOG/CFA/PTx and a relapse-remitting form induced in SJL/J (H-2^s) mice by immunization with PLP/CFA, respectively. Tolerance in both models was peptide specific and, as further studied in the MOG-EAE model, was associated with reduced antigen-specific T cell proliferation but not changes in differentiation of IFN-γ-producing Th1, IL-17-producing Th17 cells or regulatory T cells. However, we identified resistance in antigen-specific Th1 and Th17 towards CD40-mediated co-stimulatory signals from APC. This dissociation between proliferation and cytokine production in T cell responses indicates that the presentation of OM-MOG by APC results in differential signaling through the TCR on MOG-specific T cells compared to presentation of MOG, and that this results in partial T cell anergy. Indeed, $CD3^+$ T cells and activated macrophages accumulated in the leptomeninges of the spinal cord in OM-MOG-vaccinated mice after immunization with MOG, suggesting that MOG-specific T cells recognize target tissue and initiate inflammation. However, markedly reduced numbers of T cells infiltrated the CNS parenchyma to form inflammatory lesions compared to PBS-vaccinated EAE controls, and mice that developed lesions did not necessarily develop clinical signs of EAE. Overall, our results suggest that OM-MOG induces the expansion of Th1 and Th17 T cells that show impaired proliferation responses to antigen and APC-induced co-stimulatory signals that are required for licensing them to become fully pathogenic T cells.

Peripheral tolerance can be induced by via several mechanisms including T cell deletion or anergy, the induction of regulatory T cells and immune deviation (Tisch, 2010). To gain insight into the mechanism of OM-MOG-induced tolerance we monitored T cell responses to MOG stimulation *in vivo* in conjugate-vaccinated mice and *in vitro* using DC-T cell co-cultures. We found no evidence for deletion of antigen-specific T cells in the periphery of OM-MOG-vaccinated mice, as judging from the normal differentiation of IFN-γ- and IL-17-producing $CD4^+$ T cells and $FoxP3^+$ regulatory T cells isolated from secondary lymphoid organs following immunization of mice with MOG. This was supported by data from DC-T cells cultures showing that OM-MOG DC and MOG DC stimulated equal production of IFN-γ and IL-17 by $CD4^+$ 2D2 T cells, and that OM-MOG DC did not induce increased cell surface expression of the apoptosis marker annexin V compared to MOG, OM or PBS DC. Furthermore, we found no evidence for immune deviation in antigen-specific T helper cell populations and no alteration in regulatory T cell populations. We did, however, detect reduced antigen-specific T cell proliferation measured both in secondary lymphoid organs of

OM-MOG-vaccinated mice following MOG immunization, and in 2D2 T cells co-cultured with OM-MOG DC, as well as failure of OM-MOG DC-delivered co-stimulatory signals to up-regulate T cell cytokine production. The uncoupling of cytokine production from proliferation in T cells in the presence of competent APC has been described previously in response to APL (Evavold *et al.*, 1991) and during oral tolerance (Whitacre, Gienapp, Orosz, and Bitar, 1991), (Chen, Inobe, Kuchroo, Baron, Janeway, Jr., and Weiner, 1996), (Karpus, Kennedy, Smith, and Miller, 1996) and is thought to involve the differential activation of TCR signaling pathways (Sloan-Lancaster and Allen, 1996). Although the signaling pathways that underlie differential TCR-mediated effects remain to be fully elucidated, our findings add that effector T cell cytokine production, in the absence of adequate proliferative response, is not sufficient for the induction of EAE.

The mechanism by which mannan-conjugated myelin peptides reduce the encephalitogenic function of effector T cells therefore appears to be different from those previously described for immune tolerance induced by APC targeting which include association with CD5 expression (Hawiger *et al.*, 2004), reduction of IL-17 cell and increase of regulatory T cell differentiation (Stern *et al.*, 2010) and immune deviation towards an immunoregulatory profile (Apostolopoulos *et al.*, 2000), (Apostolopoulos *et al.*, 1995). It most closely resembles tolerance induced by mannosylated antigens, as previously described Nagelkerken and his group. Like mannan-MOG, mannosylated $PLP_{139-151}$ protected SJL mice against the development of PLP-EAE through an active mechanism, because it inhibited disease when administered after EAE induction by active immunization with PLP or adoptive transfer of $PLP_{139-151}$-reactive T cell blasts (Luca *et al.*, 2005), (Kel *et al.*, 2007). Also lymph node cells isolated from mice that had been immunized with mannosylated $PLP_{139-151}$ showed equal cytokine and chemokine production but reduced proliferation responses compared to cells primed with non-mannosylated $PLP_{139-151}$ (Kel *et al.*, 2008). This defect was associated with poor Th1 effector functions as shown by reduced IgG2a antibody levels, reduced DTH responses and EAE symptoms (Luca *et al.*, 2005), (Kel *et al.*, 2007). However, unlike tolerance induced by OM-MOG, which was resistant to PTx, that induced by mannosylated peptides was abrogated by PTx administration (Kel *et al.*, 2008). PTx is widely used to increase antigen-specific T cell responses and disease susceptibility in EAE models through multiple effects including prevention of antigen-induced peripheral T cell anergy (Kamradt, Soloway, Perkins, and Gefter, 1991), induction of IL-17 production (Hofstetter, Grau, Buttmann, Forsthuber, Gaupp, Toyka, and Gold, 2007) and reduction of regulatory T cells (Chen, Winkler-Pickett, Carbonetti, Ortaldo, Oppenheim, and Howard, 2006). It is possible therefore, that mannosylated and mannan-conjugated peptides induce T cell tolerance through a similar mechanism and that differences in sensitivity of the two approaches to immune adjuvants reflect differences in the strength of tolerance induced.

The MR is expressed by most tissue macrophages, other cell types including hepatic and lymphatic endothelia, a subpopulation of DC in lymphoid organs that drain the periphery and in brain meningeal macrophages and perivascular microglia in the mouse (McKenzie, Taylor, Stillion, Lucas, Harris, Gordon, and Martinez-Pomares,

2007), (Linehan *et al.*, 1999) as well as additional skin and gut DC in humans (Engering, Geijtenbeek, van Vliet, Wijers, van, Demaurex, Lanzavecchia, Fransen, Figdor, Piguet, and van, 2002). It provides an efficient internalization system for the recognition, transport and clearance of host-derived glycoproteins and microbe-derived ligands (Taylor, Martinez-Pomares, Stacey, Lin, Brown, and Gordon, 2005). Previous studies showed that engagement of the MR by mannosylated lipoarabinomannans, mannan or an anti-MR antibody inhibited LPS-induced IL-12 production by human DC (Nigou, Zelle-Rieser, Gilleron, Thurnher, and Puzo, 2001) and Toll-like receptor-dependent IL-12 production in mouse macrophage cells (Pathak, Basu, Bhattacharyya, Pathak, Kundu, and Basu, 2005). Also some, not all, natural MR ligands activated an anti-inflammatory program in human monocyte-derived DC, which included inhibition of IL-12 production and potential to polarize Th1 effector cells (Chieppa, Bianchi, Doni, Del, Sironi, Laskarin, Monti, Piemonti, Biondi, Mantovani, Introna, and Allavena, 2003). These latter studies show that appropriate engagement of the MR on DC can elicit immunosuppressive effects. To investigate the cellular basis of MOG-specific T cell tolerance in OM-MOG-vaccinated mice, we used DC cultures derived from mouse bone marrow and unexpectedly found that OM-MOG-loaded DC displayed phenotypic and functional characteristics of immune-promoting mature DC. First, DC loaded with OM-MOG or MOG efficiently presented antigen to 2D2 MOG-specific T cells and equally induced the differentiation of IFNγ-producing and IL-17-producing T cells. Second, both OM and OM-MOG increased the surface expression of CD40, CD80 and CD86 in DC to levels at least as high as LPS-treated DC, showing that mannan induces the phenotypic maturation of bone marrow-derived DC, a property consistent with its functional characteristics as a PAMP, and in agreement with our previous results on the effects of OM-MUC1 in bone marrow-derived DC as well as *in vivo* splenic and lymph node DC (Sheng *et al.*, 2006). Third, OM-MOG DC showed CD40-inducible production of the Th1 and Th17 polarizing cytokines, IL-12 and IL-23 respectively, properties that are essential for the immune-promoting functions of mature DC (Caux, Massacrier, Vanbervliet, Dubois, Van, Durand, and Banchereau, 1994), (Cella, Scheidegger, Palmer-Lehmann, Lane, Lanzavecchia, and Alber, 1996), (Koch, Stanzl, Jennewein, Janke, Heufler, Kampgen, Romani, and Schuler, 1996), (Iezzi *et al.*, 2009). These results first of all suggest that T cell tolerance induced by the administration of OM-MOG in mice is not due to an intrinsic defect in APC function. Our finding that OM-MOG induces T cell tolerance, and that *in vitro* grown OM-MOG-loaded DC are sufficient to transfer tolerance into mice with ongoing EAE therefore appears paradoxical. A number of previous studies have already challenged the concept that only immature and semi-mature DC mediate T cell tolerance by showing that phenotypically mature DC can also induce tolerance (Albert *et al.*, 2001), (Menges, Rossner, Voigtlander, Schindler, Kukutsch, Bogdan, Erb, Schuler, and Lutz, 2002), (Fujii *et al.*, 2004), (Sporri *et al.*, 2005). In some of these studies (Albert *et al.*, 2001), (Fujii *et al.*, 2004), but not all (Menges *et al.*, 2002), stimulation of CD40 changed the outcome of tolerance to immunity. Since various cell types express the MR in mice it is possible that tolerance induced by OM-MOG is mediated by APC other than DC, for example by meningeal macrophages or perivascular microglia (Linehan *et al.*, 1999) which are known to have APC properties (Greter, Heppner, Lemos, Odermatt, Goebels, Laufer, Noelle, and Becher, 2005). Nevertheless, when we administered

FGK45 *in vivo* it did not change the outcome of protection against EAE in OM-MOG-vaccinated mice while, as expected, it exacerbated disease in PBS-vaccinated mice. Taken together, our findings show that mannan-peptide-targeted APC do not show overt functional defects; on the contrary they show characteristics of mature immunogenic APC; and suggest they play a nonautonomous role in mediating the tolerogenic effects of OM-MOG to T cells.

Lymphocytes derived from OM-MOG-tolerized mice and MOG-specific T cells exposed to OM-MOG DC exhibited a selective defect in proliferation, but not cytokine responses to antigen stimulation and this unresponsiveness was overcome by exogenous IL-2. Clonal T cell anergy, characterized by reduction of antigen-specific proliferation responses but not necessarily changes in cytokine production, has been described as a mechanism of T cell tolerance induced by oral administration of MBP (Whitacre *et al.*, 1991), (Chen *et al.*, 1996) and $PLP_{139-151}$ (Karpus *et al.*, 1996), by myelin peptide-coupled splenocytes (Vandenbark, Celnik, Vainiene, Miller, and Offner, 1995) and by prostaglandin 2 (Mannie, Prevost, and Marinakis, 1995). In oral tolerance, low doses of antigen generate regulatory T cells whereas high doses induce T cell anergy or deletion. Our data suggest that OM-MOG administration in mice results in strong stimulation of self-reactive T cells by APC, through combined MOG-TCR-specific and MR-induced innate immune signals, that results in partial T cell anergy. This would be in line with our previous findings, where OM-MUC1 greatly enhanced T cell responses to MUC1 but, in that case, promoted anti-tumor immunity (Apostolopoulos *et al.*, 1995). Our finding that T cell tolerance in OM-MOG vaccinated EAE mice and OM-MOG DC-T cell cultures was not altered by CD40 co-stimulation is a strong indication of clonal T cell anergy because mice that are deficient in CD40 do not develop pathogenic Th17 T cells and are completely resistant to EAE (Becher *et al.*, 2001), (Iezzi *et al.*, 2009). It is established that *de novo* processing and presentation of CNS antigens in the context of MHC class II is absolutely required for the development of EAE (Tompkins *et al.*, 2002), (Becher, Durell, and Noelle, 2003), (Kawakami, Lassmann, Li, Odoardi, Ritter, Ziemssen, Klinkert, Ellwart, Bradl, Krivacic, Lassmann, Ransohoff, Volk, Wekerle, Linington, and Flugel, 2004) probably by APC associated with the meninges and CNS vasculature (Greter *et al.*, 2005). Previous studies showed that mannosylated PLP could ameliorate EAE when administered after the induction of EAE by adoptive transfer of activated myelin-reactive T cells (Kel *et al.*, 2007), showing that tolerance is actively induced in already activated T cells, possibly preventing their reactivation in the periphery or CNS. Similarly in this study administration of OM-MOG, either as i.d. injection or transferred with DC, actively induced tolerance in resting or already activated T cells, thereby reducing their proliferation and encephalitogenic potential and protecting mice against EAE. In line with the hypothesis that OM-MOG acts by reducing the capacity of MOG-specific T cells to be restimulated by endogenous antigen in the target tissue, is the finding that $CD3^+$ T cells accumulated in the leptomeninges and vasculature of the spinal cord and showed markedly reduced infiltration of the parenchyma after the induction of active EAE. Alternatively, it is possible that OM-MOG exerts a decoy effect, diverting the migration of MOG-specific T cells away from targets in the CNS to the site of injection in the skin, and further in vivo experiments will be needed to investigate this possibility.

Overall, mannan-conjugated peptides administered as prophylactic vaccines or therapeutically induce potent and reproducible peptide-specific protection of mice from the clinical signs of EAE with all protocols tested even in the presence of strong APC maturation stimuli. The protective effect of mannan-conjugated peptides is especially important in a setting where EAE is induced in the presence of adjuvants, like PTx and CD40, that have the ultimate role of breaking tolerance indicating that it might be a powerful strategy to silence autoimmunity in a human setting where immune challenge by microbial antigens is thought to play a key role in the breakdown of immune tolerance to self-antigens. Further studies are needed to characterize the mechanism of anergy triggered by TCR stimulation by mannan-peptide conjugates, especially since they constitute interesting targets of clinical application for therapeutic intervention in MS. Treatment with mannan-conjugated autoantigens could differ from current therapeutic regimens that are immunosuppressive and could be a step towards the design of personalized therapies for the different immunodominant peptide epitopes found in autoantigen specific immune reactions in MS and relevant demyelinating disorders.

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Abbreviations: Ag, antigen; APC, antigen-presenting cells; APL, altered peptide ligands; CFA, complete Freund's adjuvant; CNS, central nervous system; cpm, counts per minute; DC, dendritic cells; DLN, draining lymph nodes; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FCS, foetal calf serum; GA, glatiramer acetate; H&E, haematoxylin & eosin; LPS, lipopolysaccharide; MAb, monoclonal antibody; MOG, myelin oligodendrocyte glycoprotein; MR, mannose receptor; MS, multiple sclerosis; MUC1, mucin 1; OM, oxidized mannan; PAMP, pathogen-associated molecular pattern; PLP, proteolipid lipoprotein; POL, GA-type polypeptide; PTx, *Bordetella pertussis* toxin; RM, reduced mannan; RP-HPLC, reverse phase-high performance liquid chromatography; SI, stimulation index; TCR, T cell receptor; TFA, trifluoroacetic acid

Figure legends

FIGURE 1. Administration of OM-MOG or RM-MOG in prophylactic (vaccination) or therapeutic protocols attenuates the development of MOG-induced EAE in C57BL/6 mice. **(a)** Mean clinical scores of MOG-EAE in groups of mice that were vaccinated i.d. with dilute soluble OM-MOG, RM-MOG, OM, RM or PBS at indicated time points (arrows) prior to the induction of EAE by immunization with MOG/CFA/PTx (n = 6 for each group). **(b)** Mean clinical scores of MOG-EAE in groups of mice that received i.d. administration of dilute soluble peptides on day 0 and 7 relative to immunization for EAE induction (n=5 for each group). **(c)** Mean clinical scores of MOG-EAE in groups of mice injected i.d. with dilute soluble peptides at indicated time points after immunization for EAE induction (n = 5 for each group). The results shown are from one representative of two **(a, c)** or three **(b)** independent experiments. **(d, e)** Vaccination with OM-MOG protects C57BL/6 mice from spinal cord inflammation and demyelination during MOG-EAE. **(d)** Inflammatory cell infiltration was visualized by H&E (left column) and demyelination by Luxol fast blue (right column) staining of spinal cord sections taken from representative mice in the different vaccination groups on day 24 following immunization for the induction of MOG-EAE. Representative sections from 1 of 5 animals per group are shown. **(e)** Quantification of spinal cord inflammation (black bars) and demyelination (grey bars) as well as brain demyelination (white bars) in all experimental groups. Representative data from 5 animals per group are shown. Statistical significance after pair-wise comparisons of each experimental group with the non-vaccinated control (PBS) group is shown (*, $p<0.05$). Triangles (a) indicate time points where pairwise comparison between OM-MOG and RM-MOG groups also show significant differences.

FIGURE 2. Protection of mice against EAE by mannan-peptides is peptide-specific. **(a)** Vaccination of SJL/J mice with OM-$PLP_{139-151}$, but not OM-MBP_{83-99}, attenuated the development of PLP-EAE. Mean clinical scores of PLP-EAE in groups of mice that were vaccinated with OM- $PLP_{139-151}$, OM- MBP_{83-99} or PBS on the days indicated (arrows) prior to immunization for the induction of PLP-EAE (n = 5 for all groups). **(b)** Vaccination of C57BL/6 mice with OM-MOG_{35-55}, but not OM-$PLP_{178-191}$, attenuated the development of MOG-EAE. Mean clinical scores of MOG-EAE in groups of mice that were vaccinated with OM-MOG_{35-55}, OM-, $PLP_{178-191}$ or PBS on the days indicated (arrows) prior to the induction of PLP-EAE by immunization with PLP/CFA (n = 5 for all groups). Data are from one representative of two independent experiments. Statistical significance after pair-wise comparisons of each experimental group with the non-vaccinated control (PBS) group is shown (*, $p<0.05$).

FIGURE 3. Antigen-specific responses of T cells exposed to OM-MOG show reduced proliferation but normal expansion of Th1, Th17 and regulatory T cell populations. **(a)** Proliferation of splenocytes isolated from vaccinated mice 25 days post-immunization for MOG-EAE. Splenocytes were stimulated *ex vivo* with MOG (mice vaccinated with PBS n = 4; OM-MOG n = 6; RM-MOG n = 6; OM n = 5; RM n = 4). **(b)** Proliferation of CFSE-labelled MOG-specific 2D2 $CD45.1^+$ cells *in vivo* after i.v. transfer into recipient $CD45.2^+$ mice that had received a complete vaccination protocol with mannan-conjugated peptides, on day 2 after MOG-EAE induction. DLN were isolated from mice 7 days after immunization with MOG/CFA/PTx, and on the $CD45.1^+$ gate the

percentages of proliferating cells (showing low and intermediate levels of CFSE staining) were measured (mice vaccinated with MOG n = 5; OM-MOG n=5; OM n=5; PBS n=5). Right panels show representative histogram plots showing CFSE dilution in MOG-specific 2D2 CD45.1$^+$ cells in DLN isolated from OM- and OM-MOG-vaccinated mice. **(c-d)** Antigen priming of Th1 and Th17 T cells is normal in mice vaccinated with OM-MOG. Mice were vaccinated with OM, OM-MOG, MOG or a mix of unconjugated OM and MOG and immunized for the induction of MOG-EAE. **(c)** The production of IFN-γ and IL-17 by CD4$^+$ T cells from DLN isolated 7 days after MOG immunization was measured by intracellular cytokine staining (n=5 for the MOG and OM-MOG groups and n=4 for the OM and OM/MOG groups). **(d)** The production of IL-17 by CD4$^+$ T cells from DLN (left panel) and spleen (right panel) isolated on days 10 (pre-onset) and 15 (peak) of EAE was also measured by intracellular cytokine staining (n = 5 for all groups). **(e)** The production of IL-10 by CD4$^+$ T cells from DLN isolated on day 13 of EAE was also measured by intracellular cytokine staining (n = 4 for all groups). **(f)** Proportions of CD4$^+$FoxP3$^+$ cells in DLN cells from vaccinated mice 7 days post-immunization for MOG-EAE, and stained for surface CD4 and intracellular FoxP3 (n=5 mice in each group). Right panel, representative dot plots are shown from each experimental group. Data are from one (e) or one representative of two independent experiments. Statistical significance after pair-wise comparisons (using Student's t test) of each experimental group with the non-vaccinated control (PBS) group is shown.

FIGURE 4. OM-MOG-loaded DC up-regulate cell surface maturation markers, Th1 and Th17 polarising cytokines and down-regulate PD-L1 and are sufficient to transfer tolerance into EAE mice. **(a)** Flow cytometry of DC loaded with PBS or peptide conjugates or incubated with LPS (1 μg /ml), showing frequency of CD11c$^+$ cells expressing DC surface maturation markers. **(b)** Enzyme-linked immunosorbant assay of cytokines in culture supernatants of DC loaded with OM-MOG or MOG in the absence or presence of FGK 45 agonistic anti-CD40 antibody (10 μg/ml) for 72 hr. **(c)** Flow cytometry of DC loaded with PBS or peptide conjugates showing frequency of CD11c$^+$ cells expressing PD-L1 surface marker. **(d)** Mean clinical scores of mice that were immunized for the induction of MOG-EAE and 8 days later received i.v. transfer of bone marrow-derived DC loaded *ex vivo* with OM-MOG (n=9), unconjugated MOG (n = 8), OM (n = 7) or PBS (n = 8) at the indicated time point (arrow). Data are from two (**b, d**) or three (**a**) independent experiments. Statistical significance after pair-wise comparisons between groups or (**d**) or each experimental group with the non-vaccinated control (PBS) group (*, $p<0.05$) is shown.

FIGURE 5. OM-MOG DC induce normal maturation of MOG-specific Th1 and Th17 responses but reduced T cell proliferation and CD40 costimulation responses. **(a, b)** Antigen presentation assays between MOG DC and OM-MOG DC and 2D2 MOG-specific lymphocytes at a 1:5 DC:T cell ratio in the absence or presence of FGK45 agonistic anti-CD40 antibody (10 μg/ml) for 72 hr. **(a)** Proliferation of 2D2 lymphocytes by peptide-loaded DC as measured by [^{3}H] thymidine incorporation. **(b)** Production of IFN-γ and IL-17 by CD4$^+$ 2D2 T cells in response to peptide-loaded DC as measured by intracellular cytokine staining. **(c)** Annexin V$^+$ surface expression by CD4$^+$ 2D2 MOG-specific T cells stimulated by OM-MOG, MOG, OM and PBS DC. Annexin V translocation was measured at day 7 of culture. Statistical significance after pair-wise comparisons

(using Student's t test) between 2D2 cells stimulated with PBS DC versus Ag DC is shown. **(d)** Mean clinical scores of MOG-EAE in groups of mice that were vaccinated i.d. with dilute soluble OM-MOG or PBS and were further left untreated or treated by twice weekly i.p. injections of FGK45 agonistic anti-CD40 antibody (90 µg/mouse/injection) from day 2 post-immunization (n = 8 for the OM-MOG and OM-MOG + aCD40 groups; n = 4 for the PBS and the PBS + aCD40 groups). **(e)** Proliferation of 2D2 lymphocytes by peptide-loaded as measured by [^{3}H] thymidine incorporation in the absence or presence of recombinant IL-2 (10 ng/ml). Representative results from one of two independent experiments are shown. Statistical significance after pair-wise comparisons between cells or mice treated in the absence or presence of agonistic anti-CD40 antibody (a-d) (*, $p<0.05$) or IL-2 (e) is shown.

FIGURE 6. $CD3^+$ T cell and immune cell infiltration of the spinal cord in OM-MOG-vaccinated mice is sparce and limited to leptomeninges or exceptional compact white matter lesions. **(a)** Double immunofluorescence staining with anti-CD3 and anti-Ki67 antibodies identifies proliferating CD3+ T cells in spinal cord sections taken at the peak of disease from chimeric C57BL/6 mice reconstituted with bone marrow from TgEGFP mice, vaccinated with OM-MOG or OM control and immunized for MOG-EAE. Numerous CD3-immunoreactive infiltrating cells are seen in the parenchyma of PBS-vaccinated mice, many of which also show Ki67-immunoreactivity (clinical score 3.5) (upper panel, arrowheads). CD3-immunoreactive infiltrates are markedly reduced in OM-MOG vaccinated mice (clinical score 0) where they are mainly restricted to leptomeninges and the associated perivascular spaces (lower panel). **(b)** Oxidative burst in infiltrating cells shown by immunostaining of sections from the same mice as in a) with anti-p22phox (p22) and iNOS antibodies in areas of CD3+ T cell infiltration. Representative results from 5 mice per group are shown. Scale bars 500 µM (a).

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