Smad7 in T cells drives T helper 1 responses in multiple sclerosis and experimental autoimmune encephalomyelitis

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Autoreactive CD4+ T lymphocytes play a vital role in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. Since the discovery of T helper 17 cells, there is an ongoing debate whether T helper 1, T helper 17 or both subtypes of T lymphocytes are important for the initiation of autoimmune neuroinflammation. We examined peripheral blood CD4+ cells from patients with active and stable relapsing–remitting multiple sclerosis, and used mice with conditional deletion or over-expression of the transforming growth factor-ß inhibitor Smad7, to delineate the role of Smad7 in T cell differentiation and autoimmune neuroinflammation. We found that Smad7 is up-regulated in peripheral CD4+ cells from patients...
with multiple sclerosis during relapse but not remission, and that expression of Smad7 strongly correlates with T-bet, a transcription factor defining T helper 1 responses. Concordantly, mice with transgenic over-expression of Smad7 in T cells developed an enhanced disease course during experimental autoimmune encephalomyelitis, accompanied by elevated infiltration of inflammatory cells and T helper 1 responses in the central nervous system. On the contrary, mice with a T cell-specific deletion of Smad7 had reduced disease and central nervous system inflammation. Lack of Smad7 in T cells blunted T cell proliferation and T helper 1 responses in the periphery but left T helper 17 responses unaltered. Furthermore, frequencies of regulatory T cells were increased in the central nervous system of mice with a T cell-specific deletion and reduced in mice with a T cell-specific over-expression of Smad7. Downstream effects of transforming growth factor-β on in vitro differentiation of naïve T cells to T helper 1, T helper 17 and regulatory T cell phenotypes were enhanced in T cells lacking Smad7. Finally, Smad7 was induced during T helper 1 differentiation and inhibited during T helper 17 differentiation. Taken together, the level of Smad7 in T cells determines T helper 1 polarization and regulates inflammatory cellular responses. Since a Smad7 deletion in T cells leads to immunosuppression, Smad7 may be a potential new therapeutic target in multiple sclerosis.

Keywords: EAE; multiple sclerosis; immune regulation; T cell responses; T helper 1
Abbreviations: EAE = experimental autoimmune encephalomyelitis; ELISA = enzyme-linked immunosorbent assay; IFN-γ = gamma interferon; IL = interleukin; MOG = myelin oligodendrocyte glycoprotein; qRT-PCR = quantitative real-time polymerase chain reaction; RPMI = Roswell Park Memorial Institute; TGF-β = transforming growth factor-β; TGF-βR = transforming growth factor-β receptor; Th = T helper

Introduction

Multiple sclerosis is a chronic inflammatory and degenerative disease of the central nervous system (CNS) believed to be of autoimmune origin (Compston and Coles, 2008). In majority of patients, multiple sclerosis initially presents as a relapsing–remitting disease with acute inflammatory attacks against CNS tissue, followed by complete recovery. Although the exact aetiology of multiple sclerosis remains unknown, it is widely accepted that the activation of myelin-specific CD4+ T cells is a central step in the initiation of neuroinflammation (Sospedra and Martin, 2005). The strongest genetic risk factors for multiple sclerosis were found in the HLA-DRA, interleukin (IL)-2R and IL-7R genes (Hafler et al., 2007), emphasizing the importance of CD4+ T cells.

The animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), is induced by immunization with myelin components or by adoptive transfer of encephalitogenic CD4+ T cells into naïve animals (Zamvil and Steinman, 1990). Because of similarities to the EAE model, the fact that administration of the quintessential T helper (Th) 1 cytokine gamma interferon (IFN-γ) worsened multiple sclerosis (Panitch et al., 1987), and the typical cellular composition of brain- and cerebrospinal fluid-infiltrating cells, multiple sclerosis for a long time was considered to be a Th1-mediated disease (Sospedra and Martin, 2005). However, since the characterization of the Th17 phenotype and its pathogenic role in EAE (Cuadrado et al., 2003; Langrish et al., 2005; Chen et al., 2006) and multiple sclerosis (Kebir et al., 2007), there is ongoing debate whether Th1, Th17, or both subtypes of T lymphocytes are important for the initiation of autoimmune CNS inflammation (McFarland and Martin, 2007; Steinman, 2008).

Transforming growth factor (TGF)-β1 is a master regulator of immune responses, initiating signalling events in target cells that have vital and non-redundant regulatory functions (Letterio and Roberts, 1998; Li et al., 2006a). While TGF-β blocks the differentiation of Th1 and Th2 cells by inhibiting T-bet and GATA-3 (Gorelik et al., 2000, 2002), respectively, it promotes the generation of inducible regulatory T cells by up-regulating Foxp3 (Chen et al., 2003). Under inflammatory conditions, in particular in the presence of IL-6, TGF-β supports the differentiation of Th17 cells, which are implicated in autoimmune diseases (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Despite rapid progress in elucidating the cytokine networks involved in T cell differentiation, molecular mechanisms mediating context-dependent cellular responses after activation of the transforming growth factor-β receptor (TGF-βR) are only partly understood.

The Smad family of proteins mediates signalling from the TGFβR to the nucleus (Shi and Massague, 2003). Smad7, which is induced by TGFβ itself, forms part of an inhibitory feedback-loop by binding to the intracellular domain of the activated TGFβRI (Hayashi et al., 1997; Nakao et al., 1997). It prevents the phosphorylation of Smad proteins, associates with ubiquitin ligases involved in TGFβR-degradation and acts as a transcriptional repressor inhibiting Smad-dependent promoter activation (Schmierer and Hill, 2007). Since Smad7 is responsible for the fine-tuning of TGF-β signals (Itoh and ten Dijke, 2007), an aberrant expression of Smad7 might disrupt the balanced activity of TGF-β under physiological and pathophysiological conditions.

Here, we sought to address the role of Smad7 in the pathogenesis of autoimmune neuroinflammation by examining peripheral CD4+ cells from patients with multiple sclerosis and mice with either a T cell-specific deletion or over-expression of Smad7. We found Smad7 to be elevated in CD4+ cells from patients with multiple sclerosis during relapses but not remission, and a significant correlation between the expression of Smad7 and T-bet. Mice with Smad7 over-expression showed enhanced, and mice with a lack of Smad7 in T cells reduced EAE and Th1 responses. In vitro, Th17 differentiation was reciprocally regulated compared to Th1 differentiation, indicating that Smad7 controls the balance between Th1 and Th17 responses. We conclude that both initiation of EAE and relapses in multiple sclerosis are mainly driven by a
Materials and methods

Generation of Smad7fl/fl mice

The Smad7fl/fl mouse strain was generated using standard gene targeting techniques (Torres and Kuhn, 1997). In brief, a Smad7-targeting vector flanking promoter region and exon 1 (-983 to +2415) by loxp sites was generated by inserting the short arm of homology, the promoter region with exon I and the long arm of homology into the targeting vector pRP6, containing the loxp sites, the frt-flanked neomycin resistance gene, and the HSV-thymidine kinase gene as negative selection markers (Hovelmeyer et al., 2007). All DNA fragments were amplified from the C57BL/6j-derived BAC-cloned RP24-263P17 (BACPAC Resources, Children’s Hospital Oakland Research Institute) and verified by sequencing. The targeting vector was linearized and electroporated into C57BL/6j-derived embryonic stem cells (Kontgen et al., 1993). Recombinant embryonic stem cells were identified by Southern blot analysis (primers for probe: sen 5’-CGACGGAAGTGTGTC-3’ and T-bet (Hs00203436_m1). The relative mRNA amounts were determined by normalization to the glyceraldehyde 3-phosphate dehydrogenase or 18S mRNA content. The expression levels of mRNA were calculated using the ΔΔCT method and normalized to the wild-type control or human healthy donors.

Western blot

Proteins were isolated with radioimmunoprecipitation assay-buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked with 5% bovine serum albumin for 1 h at room temperature. The following primary monoclonal antibodies were used for overnight incubation: Smad2 (L6D3; Cell Signaling), phosphorylated (p-)Smad2 (Ser465/467) (138D4; Cell Signalling) and Actin (A2066; Sigma). Signals were detected by Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore) and Amersham Hyperfilm ECL (GE). The relative amount of p-Smad2 was calculated by normalizing the integrated density (volume of spot multiplied with the mean density) of p-Smad2 on the integrated density of Actin.

Flow cytometry

Flow cytometry analyses were carried out with a FACSCalibur™ flow cytometer (BD Biosciences). The following antibodies were purchased from BD Biosciences unless otherwise specified: anti-mouse CD4 (L3T4), CD8a (clone 53-6.7), CD19 (clone IM7), CD45.2 (clone 104), CD62L (clone MEL-14), CD69 (clone H1.2F3), CD90.2 (clone 53-2.1) and TCR-β (clone H57-597). Antibodies specific for mouse IL-17A (clone TC11-18H10.1), IFN-γ (clone XMG1.2) and Foxp3 (clone FJK-16s; eBioscience) were used for intracellular cytokine staining.

T cell preparation and in vitro differentiation

Naïve CD4+CD62L+ T cells were purified from spleens and lymph nodes of at least two mice per genotype with magnetic-activated cell sorting columns and magnetic beads (Miltenyi Biotec) as described (Hovelmeyer et al., 2007) or with a FACSaria™ fluorescent cell sorter (BD Biosciences). Purity of all cell preparations was measured by flow cytometry and was >95%. Isolated and pooled T cells were stimulated in complete (Kleiter et al., 2007) Roswell Park Memorial Institute (RPMI) medium (Invitrogen) with soluble anti-CD3 (clone 145.2c11, 1 µg/ml) and anti-CD28 (clone 37.51, 10 ng/ml) for the indicated times. To induce regulatory T cell polarization, TGF-β (0.4–2 ng/ml) was added, for Th1 IL-12 (10 ng/ml) and anti-IL-4 (10 µg/ml) and for Th17 TGF-β (0.4–2 ng/ml), IL-6 (20 ng/ml), anti-IFN-γ (10 µg/ml) and anti-IL-4 (10 µg/ml). For some experiments serum-free X-Vivo 15 medium (Lonza) was used. All cytokines were bought from R&D Systems and all antibodies were from BD Biosciences.

Induction and assessment of EAE

Myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (amino acid sequence: MEVGYVRSPSRSVHLRNGK) (Mendel et al., 1995) was obtained from Research Genetics. Active EAE was induced in 6- to 8-week-old mice by immunization with 50 µg of MOG(35–55) peptide emulsified in complete Freund’s adjuvant (Difco Laboratories) supplemented with 8 mg/ml of heat-inactivated Mycobacterium tuberculosis H37RA (Difco Laboratories). The emulsion was administered as a 100 µl subcutaneous injection in the tail base. Mice received 200 ng...
of pertussis toxin (Sigma Aldrich) i.p. on the day of immunization and 2 days later. Weight and clinical score were recorded daily (0 = healthy; 1 = limp tail; 2 = partial hindlimb weakness and/or ataxia; 3 = complete paralysis of at least one hindlimb; 4 = severe forelimb weakness; 5 = moribund or dead). EAE recovery was defined as an improvement of at least 1 scoring point for at least 2 days.

Histological examination
Histology was performed as described (Prinz et al., 2008). Mice were perfused during deep anaesthesia with ice-cold saline. Spinal cords were removed and fixed in 4% buffered formalin. Then, spinal cords were dissected and embedded in paraffin before staining with haematoxylin and eosin, luxol fast blue to assess the degree of demyelination, macrophage-3 antigen (BD Pharmingen) for macrophages/microglia, CD3 for T cells (Serotec), B220 for B cells (Serotec) and deposition of amyloid precursor protein, which reflects axonal damage.

Analysis of cells that infiltrate the CNS

Two weeks after immunization with MOG(35–55), mice were perfused with ice-cold phosphate buffered saline, brain and spinal cord lysed with 2 mg/ml collagenase, and infiltrating lymphocytes and resident microglia were purified by percoll gradient (detailed protocol in Greter et al., 2005). Cells were analysed by flow cytometry either immediately or after restimulation for 4 h with 50 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin and 5 μg/ml brefeldin A. The proportion of CD45<sup>high</sup>CD11b<sup>high</sup> or CD45<sup>low</sup>CD11b<sup>low</sup> infiltrating lymphocytes or CD45<sup>high</sup>CD11b<sup>high</sup> versus CD45<sup>low</sup>CD11b<sup>low</sup> resident microglia was used to calculate the number of infiltrating cells.

T cell proliferation assay

Lymph node cells and splenocytes from MOG(35–55)-immunized mice were stimulated with MOG peptide or Concanavalin (Con) A (Sigma) in 200 μl complete RPMI medium for 24–96 h in 96-well round-bottom culture plates (Kleiter et al., 2007). Cells were pulsed with 1 μCi of [3]H-thymidine (Amersham Biosciences) 16h before harvesting. Incorporation of [3]H-thymidine was measured by harvesting the cells onto glass fibre filters (Wallac Oy), followed by liquid scintillation counting.

Enzyme linked immunosorbent assay

On day 14–16 after MOG immunization, mice were sacrificed and splenocytes and lymphocytes from inguinal lymph nodes were dissected. Isolated cells were restimulated with the indicated concentration of MOG peptide or ConA for 24–96 h in complete RPMI medium. Enzyme linked immunosorbent assay (ELISA) was performed according to the manufacturer’s instructions to determine the cytokine concentrations of IFN-γ, IL-4, IL-10, IL-17 (all BD Biosciences) and TGF-β (R&D Systems) in the supernatants.

Material from patients with multiple sclerosis and healthy donors

CD4<sup>+</sup> lymphocytes were isolated from the peripheral blood of patients with relapsing-remitting multiple sclerosis diagnosed according to the McDonald criteria (Polman et al., 2005) and age- and sex-matched healthy donors. No patient had received immunosuppressive drugs or disease-modifying agents at the time of blood analysis. They were free of glucocorticosteroids for at least 2 months. Patients were considered in a stable disease course when no new neurological symptoms and no MRI activity in the 6 months before and 3 months after the study were found. Acute relapses were defined as an episode of new neurological symptoms lasting for at least 24 h; and a blood sample was taken within 4 weeks after onset of relapse. All patients were examined before glucocorticosteroid treatment. The study was approved by the Ethics Committees of the Universities of Würzburg (Study Number 399, 11/06) and Regensburg (Study Number 06/164), and informed consent was obtained from all subjects.

Statistical analysis

All experiments were analysed by ANOVA on ranks, followed by pairwise multiple comparison procedures to identify groups that were different. A value of P < 0.05 was considered statistically significant. For comparison of human Smad7- and T-bet-expression Spearman’s rank correlation was used. All statistical tests were performed with SigmaStat 3.0 software (SPSS Inc.).

Results

Generation of mice with a specific deletion of Smad7 in T cells

Smad7 is an intracellular inhibitor of TGF-β receptor signalling. Following reports indicating that TGF-β signalling plays a critical role in T cell differentiation, we were interested to investigate if the expression level of Smad7 regulates these processes. A previously reported Smad7 knockout mouse expresses a truncated Smad7 protein and exhibits a reduced birth rate (Li et al., 2006b). We therefore reasoned that complete Smad7-deficiency would lead to embryonic lethality and opted to use the Cre/loxP system to delete Smad7 in a cell-type specific manner. We constructed a targeting vector to flank a 3.4 kb fragment that contained the promoter and exon I of the smad7 gene with loxP sites (Supplementary Fig. 1A). This targeting vector was used to transfect embryonic stem cells of C57BL/6 origin (Kontgen et al., 1993). Homologous recombinant stem cell clones were tested for specific deletion of the loxP-flanked allele (Supplementary Fig. 1B) and used for blastocyst injection. Germline transmission was verified by Southern blot and PCR analysis (Supplementary Fig. 1C). After removal of the neomycin resistance cassette by Flp-mediated recombination (Rodriguez et al., 2000), mutant Smad7<sup>fl/fl</sup> mice were crossed to mice that express the Cre recombinase early in embryogenesis (Schwenk et al., 1995). The offspring, Smad7<sup>fl/+</sup> mice, were intercrossed; of 45 mice born, 29 were heterozygous for Smad7 deletion, 16 were wild-type (giving a ratio of 1.8:1), and none were homozygous, demonstrating that deletion of Smad7 leads to embryonic lethality.

Next, Smad7<sup>fl/fl</sup> mice were crossed to mice carrying the CD4Cre allele (Lee et al., 2001), resulting in CD4Cre-Smad7<sup>fl/fl</sup> mice with specific deletion of the targeted allele in CD4<sup>+</sup> cells (Supplementary Fig. 1D). Smad7<sup>fl/fl</sup> and CD4Cre-Smad7<sup>fl/fl</sup> mice
were born at the expected Mendelian frequencies and survived normally under pathogen-free conditions. qRT–PCR showed the deletion of Smad7-mRNA in purified CD4+, CD8+ and CD90.2+ cells but not in CD19+ B lymphocytes, CD11b+ myeloid cells or CD11c+ dendritic cells (Fig. 1A, Supplementary Fig. 2A and B). In addition, recombination could be excluded in CD19+ B lymphocytes and CD11c+ dendritic cells (Supplementary Fig. 2C), altogether demonstrating a T cell-specific deletion of Smad7. The TGF-β-induced increase of Smad7-mRNA was abolished in CD4+ cells lacking Smad7 (Fig. 1B), and expression of TGF-β downstream-signalling molecules such as phosphorylated Smad2 and plasminogen activator inhibitor-1 was enhanced (Fig. 1C–E). As a negative control we used T cells with a CD2-restricted Smad7 over-expression (CD2-Smad7 strain) (Dominitzki et al., 2007).

Distribution of lymphocyte populations in CD4Cre-Smad7fl/fl mice

To investigate whether deletion of Smad7 in T cells affects the homeostasis of T and B cell populations, we analysed naïve CD4Cre-Smad7fl/fl mice and littermate controls. In the thymus, the frequencies of CD4+ and CD8+ single positive as well as CD4+CD8+ double positive cells were unaltered (Fig. 2A, Supplementary Table 1). No differences were found in total numbers and ratios of CD4+/CD8+ cells and B/T cells in the spleen (Fig. 2B, Supplementary Table 2) as well as in lymph nodes (data not shown). Although the proportion of naïve CD4+CD44lowCD62Lhigh T cells, and of T cells with a memory phenotype (CD4+CD44highCD62Llow) was unchanged, we observed a small, but significant, decrease of CD4+ and CD8+ cell frequencies with an activated phenotype (CD69high) in peripheral lymphoid organs from mice where Smad7 was deleted in T cells (Fig. 2C, Supplementary Table 2).

To test whether the deletion of Smad7 affects the steady state of regulatory T cell populations, we analysed the proportion of CD4+CD25+Foxp3+ cells from the thymus and spleen. We found no differences in the frequencies of regulatory T cells in CD4Cre-Smad7fl/fl mice compared to controls (Fig. 2C, Supplementary Table 2). In summary, apart from a significant decrease in the number of activated T cells in the periphery, the lack of Smad7 in T cells did not affect the distribution of thymic or peripheral lymphocyte subsets in antigen-unchallenged mice.

Altered T cell differentiation in Smad7-deficient T cells

To determine whether the expression level of Smad7 affects T cell differentiation, we examined the generation of T cell subsets in the presence of polarizing cytokines and anti-CD3/CD28 stimulation in vitro. We found that Th1 differentiation was reduced in T cells from CD4Cre-Smad7fl/fl mice and enhanced in T cells from CD2-Smad7 mice (Fig. 3A and B). The Th1-specific transcription
Figure 2. Distribution of central and peripheral T cell populations in CD4Cre-Smad7fl/fl mice. Flow cytometry of thymocytes, splenocytes and lymphocytes obtained from 8-week-old CD4Cre-Smad7fl/fl mice and littermate controls, stained with the indicated antibodies. (A) Analysis of CD4+/CD8+ ratio from the thymus. (B) Analysis of CD4+/CD8+ ratio and T (CD90.2+)/B (CD19+) cell ratio from the spleen. (C) Proportion of naïve (CD69low) versus activated (CD69high) CD4+ and CD8+ cells from lymph nodes. (D) Analysis of CD25+Foxp3+ regulatory T cells from thymus and spleen. In all graphs, data are representative of two independent experiments with at least three mice per genotype. Data are shown for cells in the thymocyte/lymphocyte gate only (A and B), or further gated on CD4+ or CD8+ (C and D). Dead cells were excluded using Topro3. Numbers in the graphs indicate the percentage of cells from the gated population.
factor T-bet was up-regulated in T cells over-expressing Smad7, even after addition of TGF-β1. Thus, Th1 effector cells with high Smad7 levels were less prone to suppression by TGF-β1, as shown by elevated IFN-γ expression. In contrast, T cells lacking Smad7 showed a significantly enhanced suppression of T-bet- and IFN-γ-mRNA after TGF-β1-stimulation compared to Smad7 over-expressing T cells. During Th17 differentiation, which depends on the cytokines IL-6 and TGF-β (Veldhoen et al., 2006), Smad7-deficient T cells produced more IL-17A compared to wild-type T cells, while Smad7 over-expressing T cells produced less. Similar observations were made for mRNA expression of IL-17A and of the specific transcription factor RORγt. Next, we examined differentiation of induced regulatory T cells and found an increased expression of the specific transcription factor FoxP3.

Figure 3  Smad7-deficiency increases TGF-β responses during in vitro T cell differentiation. Naïve CD4+CD62L+ T cells were obtained from 8-week-old wild-type (WT), CD4Cre-Smad7fl/fl and CD2-Smad7 mice. (A) T cells were incubated under Th1, Th17 and regulatory T cell (Treg) differentiation conditions for 5 days and stained with the indicated antibodies. Before staining for IL-17A and IFN-γ, cells were triggered with phorbol 12-myristate 13-acetate/ionomycin and golgi stop for 4 h. Data are shown for cells gated on CD4+ lymphocytes, excluding dead cells using Topro3. Numbers in the graphs indicate the percentage of cells from the gated population. One representative example of three to five independent experiments is shown. (B) qRT-PCR of transcription factors and cytokines specific for Th1, Th17 and regulatory T cell lineage polarization. Naïve T cells were purified and incubated for 5 days under differentiation conditions with or without 2 ng/ml TGF-β1 (n = 2–3). Shown is the expression relative to 18S-RNA. (C) ELISA measuring IL-10 secretion of T cells after stimulation in Th17 polarizing conditions for 5 days (n = 5). (D) ELISA measuring TGF-β1 secretion (n = 3). Purified CD4+ cells were stimulated in serum-free medium with anti-CD3/CD28 and IL-2 (10 ng/ml) for 72 h. (E) qRT-PCR showing a time kinetic of Smad7, specific transcription factors and cytokines during Th1 and Th17 differentiation of wild-type T cells (n = 3). Expression is presented relative to 18S-RNA and normalized on the unstimulated control (=1.0 arbitrary units). (B–E) Bars show mean ± SEM of independent experiments. *P<0.05 (ANOVA).
in the absence of Smad7 expression and a corresponding decrease in Smad7-over-expressing T cells. Since sustained TGF-β stimulation drives IL-10 production in Th17 cells (McGeachy et al., 2007), we also examined IL-10 secretion by Th17 differentiated T cells. Although considerable amounts of IL-10 were secreted with a rising TGF-β concentration, no significant difference was found in Smad7-deficient T cells compared to wild-type T cells, but Smad7-over-expressing T cells secreted less IL-10 (Fig. 3C).

Secretion of IL-9 was below detection limit in Smad7-deficient T cells compared to wild-type T cells, but partially served as a source of TGF-β in Smad7-deficient versus over-expressing T cells during Th17 differentiation (data not shown).

To investigate whether TGF-β produced by the T cells contributed to the changes in T cell differentiation, we stimulated purified T cells with anti-CD3/CD28 and IL-2 under serum-free conditions and measured TGF-β1 secretion by ELISA. Indeed, T cells from \textit{CD4Cre-Smad7fl/fl} mice produced significantly higher levels of TGF-β1 than T cells from \textit{CD2-Smad7} mice and thus could potentially serve as a source of TGF-β (Fig. 3D).

Finally, we tested the hypothesis that Smad7-expression is altered during T cell differentiation to enhance or inhibit polarization under the control of TGF-β. It has been shown before that inducible regulatory T cells down-regulate Smad7 by FoxP3-mediated inhibition of Smad7 promoter activity (Fantini et al., 2004). We found a significant reduction of Smad7-mRNA expression during Th17 differentiation of wild-type T cells and an increase during Th1 differentiation (Fig. 3E). Interestingly, the change in the expression of Smad7 preceded up-regulation of the lineage-specific transcription factor and effector cytokine in Th17, but not in Th1 polarization.

**Decreased EAE in CD4Cre-Smad7\textsc{fl/fl} mice**

Th1, Th17 and regulatory T cells were shown to play important roles in autoimmune brain inflammation (McFarland and Martin, 2007). To test if deletion of Smad7 affects inflammation \textit{in vivo}, we immunized \textit{CD4Cre-Smad7\textsc{fl/fl}} mice as well as \textit{Smad7\textsc{fl/+}}, Smad7\textsc{del+} and \textit{CD4Cre-Smad7\textsc{del/+}} littermate controls with the MOG 35–55 peptide and examined disease progression by clinical evaluation. The incidence of EAE was reduced by \textasciitilde40% in \textit{CD4Cre-Smad7\textsc{fl/fl}} mice (Fig. 4A, Table 1). Signs of paralysis in sick \textit{CD4Cre-Smad7\textsc{fl/fl}} mice started slightly earlier as compared to control animals, indicating that early events in EAE pathogenesis, such as recruitment of inflammatory cells, might be enhanced in the absence of Smad7 expression in T cells. However, \textit{CD4Cre-Smad7\textsc{del/+}} mice did not develop severe EAE, but rather milder clinical signs of the disease. Furthermore, most diseased animals in that group recovered earlier than control mice (after 5.4 \pm 1.02 SD days).

To investigate T cell proliferation, we isolated T cells of \textit{CD4Cre-Smad7\textsc{fl/fl}} mice and littermate controls 15 days after immunization with MOG(35–55). Upon restimulation with MOG-peptide, Smad7-deficient T cells proliferated significantly less than control T cells (Fig. 4B). Smad7-deficient T cells also proliferated less when stimulated with Concanavalin A.

Next, we analysed histological samples from brain and spinal cord tissue obtained 28 days after induction of EAE. In correlation with the clinical disease scores, we found less inflammation, demyelination and axonal damage in \textit{CD4Cre-Smad7\textsc{fl/fl}} mice as compared to control mice (Fig. 4C). Detailed analysis revealed reduced numbers of infiltrating mononuclear cells such as invading CD3+ T cells, macrophage-3 antigen<sup>+</sup> macrophages/microglia and B220<sup>+</sup> B cells (Fig. 4D). Furthermore, demyelination and deposition of amyloid precursor protein, a marker for axonal damage, were reduced.

**Th1 responses are reciprocally altered during EAE in mice with T cell-specific deletion and over-expression of Smad7**

To confirm our \textit{in vivo} data indicating that Smad7 expression in T cells contributes to the development of EAE, we used a second mouse model with T cell-specific over-expression of Smad7. After immunization with MOG(35–55), \textit{CD2-Smad7} mice showed a normal onset of disease at Day 10, illustrating that the priming phase of EAE was unaltered (Fig. 5A, Table 1). The clinical course of EAE in \textit{CD2-Smad7} mice inversely correlated with \textit{CD4Cre-Smad7\textsc{fl/fl}} mice and disease was more severe compared to wild-type mice, with a significantly increased mean maximum disease score and a mortality of 30% occurring 4.3 \pm 0.47 days after onset of disease. Prior to death, \textit{CD2-Smad7} mice showed typical EAE with progressive ascending paralysis but no clinical signs of an exaggerated systemic inflammatory response. In contrast to \textit{CD4Cre-Smad7\textsc{fl/fl}} mice, the proliferation of splenocytes obtained from \textit{CD2-Smad7} mice was not altered compared to wild-type controls (Supplementary Fig. 3A).

To investigate the phenotype and migration of activated T cells to the CNS during EAE, we isolated CNS infiltrating cells of mice 15 days after immunization with MOG(35–55). In accordance with an ameliorated clinical disease score in \textit{CD4Cre-Smad7\textsc{fl/fl}} mice, there was a significant reduction in the number of infiltrating CD45<sup>+</sup>CD11b<sup>+</sup> total lymphocytes and CD4<sup>+</sup>CD45<sup>+</sup>CD11b<sup>+</sup> T cells versus CD45<sup>+</sup>CD11b<sup>+</sup> CNS-resident cells compared to wild-type controls (Fig. 5B and C). In contrast, \textit{CD2-Smad7} mice showed significantly enhanced numbers of infiltrating total lymphocytes and CD4<sup>+</sup> T cells during acute EAE compared to \textit{CD4Cre-Smad7\textsc{fl/fl}} mice. Whereas the frequencies of inflammatory Th1 and Th17 cells in \textit{CD4Cre-Smad7\textsc{fl/fl}} mice were slightly lower compared to wild-type controls at Day 15 after immunization, \textit{CD2-Smad7} mice showed a significant increase of Th1 cells and a trend towards less Th17 cells in the inflamed CNS (Fig. 5B and D). Finally, numbers of infiltrating regulatory T cells in the CNS were reduced in \textit{CD2-Smad7} mice and increased in \textit{CD4Cre-Smad7\textsc{fl/fl}} mice.

Next, we investigated whether during antigen restimulation cytokine secretion and T cell differentiation were differentially regulated in the experimental groups. Interestingly, we found an inverse correlation of Th1- and Th17-inhibition in Smad7-deficient versus over-expressing T cells (Fig. 5E and F). Whereas \textit{CD4Cre-Smad7\textsc{fl/fl}} splenocytes secreted significantly less IFN-γ, \textit{CD2-Smad7} splenocytes secreted less IL-17A.
Figure 4  Reduced EAE in mice with a T cell-specific deletion of Smad7. (A) Clinical scores of MOG(33–55)-induced EAE for mice (n=5) with a T cell-specific Smad7 deletion (CD4Cre-Smad7fl/fl) and littermate controls (Smad7fl/fl, Smad7fl/+). The difference in mean clinical scores for the CD4Cre-Smad7fl/fl and the control groups was statistically significant from Day 14 to 27 (P<0.05, ANOVA). Results are presented as mean values ± SEM and are representative of two different experiments. (B) Proliferation assay of splenocytes obtained at Day 15 after immunization and restimulated with increasing concentrations of MOG(35–55) (left) or Con A (right) for 96 h. Proliferation was measured by thymidine-incorporation. Data are expressed as the mean ± SEM of three independent experiments. *P<0.05; **P<0.005 compared to Smad7fl/+ (ANOVA). (C) Immunohistochemistry of paraffin-embedded spinal cord sections from paraformaldehyde-perfused Smad7fl/fl, Smad7fl/+ and CD4Cre-Smad7fl/fl mice on Day 28 after induction of EAE with MOG(35–55). Sections were stained with haematoxylin and eosin, luxol fast blue-periodic acid Schiff (LFB), anti-macrophage-3 antigen (Mac-3), anti-CD3, anti-B220 or anti-amyloid precursor protein to visualize axonal damage. Shown are sections of representative mice. Arrows indicate positive cells. Scale bar 100 μM. (D) Summary of experiment shown in (C), the horizontal line represents the mean of all mice per group. ANOVA was used for statistical analysis; *P<0.05 (Smad7fl/+ versus CD4Cre-Smad7fl/fl).
correlates with T-bet during active multiple sclerosis and during EAE and upon antigen restimulation CD4+ cells lacking CD4Cre-Smad7fl/fl T-bet were up-regulated in (Fig. 5H). Whereas transcripts for the Th1 transcription factor were found in CD4Cre-Smad7fl/fl cells was found in cytes contributes to human autoimmune CNS inflammation as compared to controls. Moreover, there was an increase of IFN-γ secretion by Smad7-over-expressing T cells after 24 h resti- mulation with anti-CD3/CD28, consistent with a decrease of IFN-γ produced by Smad7-deficient T cells (Fig. 5G). Splenocytes obtained from CD4Cre-Smad7fl/+ and CD2-Smad7 mice did not secrete IL-4 (data not shown) and only low amounts of IL-10 (Supplementary Fig. 3B) during antigen restimulation.

To test whether over-expression of Smad7 in peripheral lympho- cytes contributes to human autoimmune CNS inflammation as compared to controls, we performed qRT–PCR analysis of RNA extracted from peripheral CD4+ cells obtained at the peak of acute disease (Fig. 5H). Whereats transcripts for the Th1 transcription factor T-bet were up-regulated in CD2-Smad7 mice, no major changes were observed for transcription factors of the Th2 (GATA3) and regulatory T cell (FoxP3) lineages in CD4Cre-Smad7fl/+ and CD2-Smad7 mice. Th17-specific transcripts (IL-17A, IL-17F) were down-regulated in CD4Cre-Smad7fl/+ and CD2-Smad7 mice. Notably, mRNA coding for IL-10, whose expression is increased in Th17 cells with a regula-tory phenotype (McGeachy et al., 2007), was also up-regulated in CD4+ cells lacking Smad7. Thus, similar to in vitro differentiation, during EAE and upon antigen restimulation CD4+ cells lacking Smad7 differentiated less, and CD4+ cells with Smad7 over-expression differentiated more to a Th1 phenotype, whereas Th17 differentiation was reciprocally altered.

To test if peripheral T cell populations, in particular regulatory T cells, were altered after EAE induction in CD4Cre-Smad7 mice, we examined the distribution of T cell subpopulations in the spleen and draining lymph nodes 14 days after immunization with MOG(35–55). The total T cell numbers as well as the ratio of CD4+CD8− cells and naïve (CD4+CD44+CD62Lhigh) versus memory (CD4+CD44hiCD62Llow) T cells did not differ between CD4Cre-Smad7fl/+ and control mice (Supplementary Fig. 3C and D). Moreover, no change in the number of peripheral regulatory T cells was found in CD4Cre-Smad7fl/+ and CD2-Smad7 mice (Supplementary Fig. 3E).

### Table 1 Clinical features of MOG(35–55)-induced EAE

| Genotype | Incidence | EAE-related deaths | Day of onset | Maximum clinical score |
|----------|-----------|--------------------|--------------|------------------------|
| Smad7fl/+ | 26 of 26 (100%) | 0 of 26 | 11.19 ± 2.27 | 3.17 ± 0.80 |
| Smad7fl/fl | 37 of 41 (90.2%) | 0 of 41 | 12.11 ± 2.26 | 2.56 ± 1.26 |
| CD4Cre-Smad7fl/+ | 8 of 8 (100%) | 0 of 8 | 10.75 ± 1.56 | 3.00 ± 0.61 |
| CD4Cre-Smad7fl/fl | 19 of 34 (55.9%) | 0 of 34 | 9.79 ± 1.96 | 1.25 ± 1.32*** |
| CD2-Smad7 | 10 of 10 (100%) | 3 of 10 | 10.60 ± 1.43 | 4.25 ± 0.64§ |

Smad7fl/+ and CD4Cre-Smad7fl/+ indicate the control mice (no homozygous Cre-mediated deletion of Smad7), CD2-Smad7 the Smad7-over-expressing control. Results are presented as mean ± SD and show the total number of individual mice within two (CD4Cre-Smad7fl/+ and CD2-Smad7), five (Smad7fl/+), or seven (Smad7fl/fl, CD4Cre-Smad7fl/+ independent experiments. ANOVA was used for statistical analysis. *P < 0.05 compared to Smad7fl/+ and Smad7fl/fl, **P < 0.001 compared to all other genotypes, P < 0.001 compared to Smad7fl+, Smad7fl/fl and CD4Cre-Smad7fl/fl.

To analyse T cell differentiation further during EAE in these mice, we measured the ratio of IFN-γ secretion by Smad7-over-expressing T cells after 24 h resti-mulation (Supplementary Fig. 3E).

Smad7 is up-regulated in CD4+ T cells during active multiple sclerosis and correlates with T-bet

To test whether over-expression of Smad7 in peripheral lymphocytes contributes to human autoimmune CNS inflammation as seen in CD2-Smad7 mice, we isolated CD4+ cells from patients with relapsing remitting multiple sclerosis (Supplementary Table 3) during relapse (n = 16) and analysed Smad7-mRNA expression by qRT–PCR. Indeed, we found a significant increase of Smad7 expression during active multiple sclerosis, but not during remis-sion (n = 15) or in healthy controls (n = 15) (Fig. 6A). Furthermore, there was a high correlation (R = 0.81) of Smad7 expression with expression of the Th1-specific transcription factor T-bet (Fig. 6B), suggesting that during relapses of multiple sclerosis TGF-β signaling in peripheral CD4+ cells is inhibited and hence a Th1 response predominates.

**Discussion**

In this work, we have investigated how an intracellular signalling inhibitor of TGF-β regulates T cell differentiation in vitro, during EAE and in patients with multiple sclerosis. We found a significantly increased expression level of Smad7 in CD4+ cells from patients with acute relapses as compared to remission and healthy controls. Furthermore, expression of Smad7 highly correlated with that of T-bet, a transcription factor defining Th1 responses (Szabo et al., 2000). Using mutant mice we demonstrate that the expres-sion level of Smad7 in T cells dictates whether a Th1 or a Th17 response evolves during T cell differentiation in vitro and after immunization of mice with an encephalitogenic peptide. Since susceptibility to EAE was associated with elevated Smad7 and a predominant Th1, but not Th17 polarization, we propose that both initiation of EAE and relapses in multiple sclerosis are mainly T-bet-/Th1-driven.

After characterization of IL-17 producing effector T cells, it was suggested that Th17 are the main pathogenic population causing EAE (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). By showing that CD4Cre-Smad7fl/+ mice have mild disease despite a normal peripheral Th17 response and CD2-Smad7 mice have severe disease despite a decrease of Th17 differentiation in the periphery and the CNS, we demonstrate that a Th1 response is not only sufficient, but necessary to induce EAE, and present further evidence that IL-17 producing cells are dispensable for EAE induction (Haak et al., 2009). As a note of caution, Smad7-deficient Th17 cells may be phenotypically or functionally different from wild-type Th17 cells.

Recent reports suggested that organ-specific autoimmune inflammation can be induced by both, polarized Th17 as well as
Th1 cell lines and that the conditions present during the initial antigen exposure determine the effector phenotype (Kroenke et al., 2008; Lugerv et al., 2008; Steinman, 2008). However, it was demonstrated that only Th1 cell lines can consistently induce EAE and that the encephalitogenicity of Th17 cells depends on activation of the transcription factor T-bet (Yang et al., 2009). Accordingly, T-bet deficient mice are resistant to various allergic and autoimmune diseases, including EAE (Nath et al., 2006).

We have shown that during in vitro differentiation and in the course of EAE Smad7-expression regulates the generation of Th1 cells, which are known to express high levels of Smad7 (Veldhoen et al., 2006). Smad7 may be an early marker of Th1 polarization, since both Smad7 (Ulooa et al., 1999) and T-bet (Afkarian et al., 2002) are activated by the transcription factor signal transducer and activator of transcription (STAT)-1. It has been shown that proinflammatory cytokines like IFN-γ and TNF-α up-regulate Smad7 through STAT1, which allows a Th1 response to proceed without suppression by TGF-β (Ulooa et al., 1999; Bitzer et al., 2000). Indeed, lymphocytes up-regulate Smad7 during antigen restimulation and inhibition of Smad7 by specific antisense oligonucleotides reduces T cell proliferation, the clinical score and neuroinflammation during EAE (Kleiter et al., 2007). Our data confirm that Smad7 is a critical factor in the generation of Th1 cells, but it remains to be determined if Smad7 interacts with or regulates signalling pathways or transcription factors involved in Th1 differentiation.

It was shown that Smad7 expression is absent in T cells from multiple sclerosis patients with a stable disease course and increase in the frequency of peripheral regulatory T cells (Correale and Farez, 2007). Contrary to Th1 cells, regulatory T cells down-regulate Smad7-mRNA (Mizobuchi et al., 2003; Fantini et al., 2004). The proinflammatory cytokine IL-6 blocks regulatory T cell generation by transactivation of Smad7-mRNA and CD4+CD25+ T cells from CD2-Smad7 mice are resistant to the induction of Foxp3 (Dominitzki et al., 2007). We found that Foxp3 expression was enhanced under in vitro conditions in Smad7-deficient and reduced in Smad7-over-expressing T cells. In vivo, we found the number of CNS infiltrating regulatory T cells to be reduced in CD2-Smad7 compared to CD4Cre-Smad7fl/fl mice. In contrast, regulatory T cell frequencies in the periphery were unaltered. Enhanced frequencies of regulatory T cells in the CNS may have contributed to the reduction of total lymphocytes as well as Th1 and Th17 effector T cells in the CNS of CD4Cre-Smad7fl/fl mice.

TGF-β controls T cell homeostasis at multiple checkpoints including T cell activation, proliferation, differentiation and apoptosis (Li et al., 2006a). Many immune cells are responsive to TGF-β (Letterio and Roberts, 1998), but it is now apparent that, in particular, the loss of TGF-β signalling in T cells results in the breakdown of tolerance and autoimmunity. Contrary to mice with TGF-βR-deficiency in T cells, which show T cell activation, expansion of autoreactive T cells and reduced numbers of regulatory T cells (Li et al., 2006c; Marie et al., 2006), mice lacking Smad7 in T cells exhibited a normal distribution of peripheral T cell subsets and a reduction in CD69+ activated T cells. Smad7-deficient T cells proliferated less during antigen-specific and polyclonal restimulation. Although it is highly likely that also in vivo Smad7-deficient T cells proliferate less in situations where TGF-β is present, a T cell intrinsic defect or compromised antigen presentation could have added to reduced T cell activation in CD4Cre-Smad7fl/fl mice. We did, however, exclude a deletion of Smad7 in antigen presenting cells as a confounding factor. Interestingly, Smad7-deficient T cells produced more TGF-β upon anti-CD3/28 stimulation compared to controls, which may have enhanced the inhibitory effects on Th1 differentiation, acting through auto- or paracrine mechanisms. Finally, it is conceivable that deletion of Smad7 also rendered CD8+ T cells more susceptible to the effects of TGF-β, which could have altered their effector functions (Li et al., 2006a). Thus, insufficient T cell activation as well as tolerance induction through TGF-β may have contributed to the deceased autoimmune neuroinflammation in CD4Cre-Smad7fl/fl mice.

We demonstrate up-regulation of Smad7-mRNA in T cells of patients with active multiple sclerosis, which highly correlated with the expression of T-bet. Similar to CD2-Smad7 mice, a predominance of Th1 responses in the periphery during multiple sclerosis relapses is likely. Indeed, in patients with relapsing-remitting multiple sclerosis increased expression of pSTAT1 and T-bet in peripheral CD4+ and CD8+ cells was shown to correlate with clinical and magnetic resonance imaging activity (Frisullo et al., 2006) as well as expression of T-bet in peripheral blood mononuclear cells with response to interferon-beta treatment (Drulovic et al., 2009). Enhanced type I interferon responses in the serum have been reported in a subset of patients with multiple sclerosis (Degre et al., 1976; van Baarsen et al., 2006; Comabella et al., 2009) and in subjects with other inflammatory diseases like Sjögren’s syndrome (Emamian et al., 2009), rheumatoid arthritis (van der Pouw Kraan et al., 2007) and systemic lupus erythematosus (Baechler et al., 2003), and may be a general mechanism of autoimmunity. Notably, the IRF8 locus, which is involved in the regulation of responses to type I interferons, was recently identified as a new susceptibility allele for multiple sclerosis (De Jager et al., 2009).

Others have found that both Th1 and Th17 responses are elevated in peripheral CD4+ cells during relapses (Fransson et al., 2009; Kebir et al., 2009), leaving the possibility that either Smad7 is over-expressed only in a subset of CD4+ cells, that Smad7 expression in peripheral CD4+ cells is transient, or that CD4+ cells secreting both IFN-γ and IL-17 are generated in the presence of elevated Smad7 expression. Interestingly, mononuclear cells in the cerebrospinal fluid from patients with multiple sclerosis show elevated IL-17 mRNA expression and a relative increase of IL-17 expressing cells compared to blood (Matusевичius et al., 1999; Brucklacher-Waldert et al., 2009). Furthermore, significant numbers of perivascular dendritic cells capable of driving Th17 differentiation and of intralosomal IL-17 producing CD4+ cells were found in patients with multiple sclerosis (Ifergan et al., 2008; Tzartos et al., 2008). Hence, unlike the situation in the periphery, Th17 cells may be the predominant effector T cell population in the CNS of patients with multiple sclerosis. Finally, multiple sclerosis lesions show a gene expression profile of IL-6, IL-17, osteopontin and of IFN-driven transcriptional activity (Chabas et al., 2001; Lock et al., 2002).
Figure 5  Reciprocally altered Th1 and Th17 responses during EAE in mice with T cell-specific deletion and over-expression of Smad7. (A) Clinical scores of MOG(33-55)-induced EAE for mice (n = 10) with a T cell-specific Smad7 deletion (CD4Cre-Smad7fl/fl), littermate controls (Smad7fl/+), or a T cell-specific over-expression of Smad7 (CD2-Smad7). The difference in clinical scores for CD4Cre-Smad7fl/fl versus CD2-Smad7 mice was statistically significant from Day 12 to 16 (P<0.001, ANOVA). Results are presented as mean values ± SEM. Pooled mice from two experiments are shown. (B) Mice were immunized with MOG(35–55) and CNS infiltrating cells isolated at Day 15. The first panel shows the number of CD45.2highCD11blow infiltrating lymphocytes (R1) and CD45.2highCD11bhigh infiltrating macrophages.
In summary, we have shown that Smad7, which is critically involved in TGF-β signalling, is a major regulator of T cell differentiation and autoimmune CNS inflammation that governs the Th1/Th17 decision. Increased Smad7 expression in CD4+ cells was found during active multiple sclerosis and aggravated clinical disease and Th1 responses in the EAE model. Lack of Smad7 in T cells ameliorated EAE and blunted Th1 effector mechanisms. Targeting of Smad7 might therefore be a valuable therapeutic approach for T cell-mediated autoimmune diseases of the CNS.

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Supplementary material

Supplementary material can be found at Brain online.

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