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Pharmacological prion protein silencing accelerates central nervous system autoimmune disease via T cell receptor signalling

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The primary biological function of the endogenous cellular prion protein has remained unclear. We investigated its biological function in the generation of cellular immune responses using cellular prion protein gene-specific small interfering ribonucleic acid *in vivo* and *in vitro*. Our results were confirmed by blocking cellular prion protein with monovalent antibodies and by using cellular prion protein-deficient and -transgenic mice. *In vivo* prion protein gene-small interfering ribonucleic acid treatment effects were of limited duration, restricted to secondary lymphoid organs and resulted in a 70% reduction of cellular prion protein expression in leukocytes. Disruption of cellular prion protein signalling augmented antigen-specific activation and proliferation, and enhanced T cell receptor signalling, resulting in zeta-chain-associated protein-70 phosphorylation and nuclear factor of activated T cells/activator protein 1 transcriptional activity. *In vivo* prion protein gene-small interfering ribonucleic acid treatment promoted

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T cell differentiation towards pro-inflammatory phenotypes and increased survival of antigen-specific T cells. Cellular prion protein silencing with small interfering ribonucleic acid also resulted in the worsening of actively induced and adoptively transferred experimental autoimmune encephalomyelitis. Finally, treatment of myelin basic protein$_{1–11}$ T cell receptor transgenic mice with prion protein gene-small interfering ribonucleic acid resulted in spontaneous experimental autoimmune encephalomyelitis. Thus, central nervous system autoimmune disease was modulated at all stages of disease: the generation of the T cell effector response, the elicitation of T effector function and the perpetuation of cellular immune responses. Our findings indicate that cellular prion protein regulates T cell receptor-mediated T cell activation, differentiation and survival. Defects in autoimmunity are restricted to the immune system and not the central nervous system. Our data identify cellular prion protein as a regulator of cellular immunological homoeostasis and suggest cellular prion protein as a novel potential target for therapeutic immunomodulation.

Keywords: small interfering RNA; prion; immunosuppression; T cell signaling

Abbreviations: AP-1 = activator protein 1; EAE = experimental autoimmune encephalomyelitis; ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; IL = interleukin; MBP = myelin basic protein; NFAT = nuclear factor of activated T cells; PBS = phosphate buffered saline; PMA = phorbol 12-myristate 13-acetate; PrP = prion protein; *Prnp* = prion protein gene; siRNA = small interfering ribonucleic acid; TCR = T cell receptor; Th = T helper cell; Zap70 = zeta-chain-associated protein-70

Introduction

A physiological function for cellular prion protein (PrP^c), a glycophosphatidylinositol-anchored sialoglycoprotein, is still enigmatic. So far, PrP^c has mainly been studied in the realm of neurodegenerative diseases, specifically as the substrate for the replicative cycle of prions. PrP^c is highly expressed in neurons (DeArmond *et al*., 1999). However, high expression levels of PrP^c in lymphoid cells (Cashman *et al*., 1990; Antoine *et al*., 2000; Durig *et al*., 2000; Li *et al*., 2001) and myeloid cells (Burthem *et al*., 2001; Krebs *et al*., 2006) suggest a biological function of PrP^c outside the CNS. The recently reported localization of PrP^c in the immunological synapse, and its co-precipitation with the T cell receptor (TCR) (Mattei *et al*., 2004) prompted us to investigate more closely the role of PrP^c in mediating T cell activation and function. The role and function of T cells in neuroinflammation can be paradigmatically examined in experimental autoimmune encephalomyelitis (EAE), an animal model of the human disorder multiple sclerosis.

Here, we silenced the PrP gene (*Prnp*) product with small interfering ribonucleic acid (siRNA). We observed a significant and selective decrease of PrP^c expression in lymphoid tissue, but not in the CNS. Also, silencing of PrP^c resulted in a significant worsening of clinical EAE. This finding was confirmed in genetic PrP^c-deficient mice (Manson *et al*., 1994) whereas mice overexpressing PrP^c (Fischer *et al*., 1996) were relatively resistant to EAE induction. Finally, we were able to show that PrP^c silencing in T lymphocytes accounted for the observed clinical effects through enhanced T cell activation, TCR signalling and propagation of T cell differentiation towards proinflammatory T helper cell (Th)-1 and Th17 phenotypes.

Materials and methods

Peptides and antibodies

The siRNAs were purchased as purified duplexes from Dharmacon RNA Technologies:
Prnp-SiRNA 5′-GUGCACGACUCAAUAdT dT; 3′-dTdTCACGUGCUGA CGCAGUUAU-5′ and nonsense siRNA 5′-CGAACGAGUACCGUA CACUdTdT; 3′-dTdTGCUUGCUCAUGGCAUGUGA-5′. myelin oligodendrocyte glycoprotein $(MOG)_{p35–55}$, myelin basic protein $(MBP)_{Ac1–11}$, proteolipid protein $(PLP)_{p139–151}$ and ovalbumine $(OVA)_{p323–339}$ were synthesized by S.C. Bio. Rabbit anti-mouse PrP^c monoclonal antibody ab52604 was obtained from Abcam Inc. Anti-phosphorylation clone 4G10 (Upstate), rabbit anti-mouse β-actin, goat anti-mouse Immunoglobulin G (IgG)-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc. Hamster anti-mouse CD3 (145-2C11) and hamster anti-mouse CD28 (37.51) monoclonal antibodies were purchased from BD Biosciences. The anti-PrP monoclonal antibody W226 was derived from PrP^c-deficient mice (Bueler *et al*., 1992) immunized with purified PrP^{Sc} (Petsch and Stitz, manuscript in preparation). The monoclonal antibody 19C4 was derived from $PrP^{c-/-}$ mice immunized with recombinant mouse PrP^c. The single-chain variable fragment was generated from W226 hybridoma (Muller-Schiffmann *et al*., 2009).

Mice

C57BL/6 and Sv129 mice (8- to 12-week-old) were bred at the animal-breeding facilities at the Friedrich-Loeffler-Institute in Tübingen, Germany. $OVA_{323–339}$ TCR transgenic (OT2) mice were purchased at The Jackson Laboratories. SJL/J and B10.PL mice were purchased from the Jackson Laboratory (Bar Harbor) and bred in a barrier animal facility at the University of Texas Southwestern Medical Centre. $PrP^{-/-}$ mice were made available to us by Dr. Jean Manson (Manson *et al*., 1994). Tga20 mice (Fischer *et al*., 1996) were made available by Dr Adriano Aguzzi, and bred at the Friedrich Loeffler Institute at Tübingen. B10.PL mice transgenic for $MBP_{1–11}$-specific TCR were a gift from Dr J. Goverman and Dr J. Lafaille. All protocols involving mice handling were approved by the respective Institutional Animal Care and Use Committee.

Experimental autoimmune encephalomyelitis

EAE was induced in 8- to 12-week-old female SJL/J mice by subcutaneous (s.c.) immunization with 100 μg of $PLP_{p139–151}$, which was dissolved in complete freund's adjuvant containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) as described

(Stuve *et al*., 2002, 2006). Immediately thereafter and again 48 h later, mice received an intraperitoneal (i.p.) injection of 400 ng pertussis toxin (Ptx) in 0.2 ml of phosphate buffered saline (PBS). For adoptive transfer experiments, SJL/J mice were sacrificed at Day 10 after immunization with $PLP_{p139-151}$. Splenocytes and lymph node cells were isolated and transfected with *Prnp*-siRNA or nonsense-siRNA, and then cultured in the presence of $PLP_{p139-151}$ and interleukin (IL)-12 for 72 h. Viable cells were counted and 2.5 million T cell blasts were injected i.p. into naive SJL/J recipient mice. At the time of adoptive transfer, the recipient mice were treated with *Prnp*-siRNA or nonsense-siRNA. Mice were examined daily for clinical signs of EAE and scored as previously described (Stuve *et al*., 2002, 2006): 0 = no paralysis; 1 = loss of tail tone; 2 = hindlimb weakness; 3 = hindlimb paralysis; 4 = hindlimb and forelimb paralysis; 5 = moribund or dead.

Histopathology

Mice were anaesthetized and perfused transcardially with PBS and 4% paraformaldehyde. Brains and spinal cords were dissected and embedded in paraffin. Inflammation was assessed by haematoxylin and eosin staining at Day 29 after disease induction in $Prp^{-/-}$ ($n = 6$) and wild-type mice ($n = 7$) and expressed as the mean number of inflammatory infiltrates per spinal cord cross-section (inflammatory index). A minimum of 12 spinal cord cross-sections were examined per animal. Brains and spinal cords of siRNA-treated animals were obtained from three mice with EAE of each experimental group at Day 20 and were evaluated by an examiner, blinded to the treatment status of the animal. Quantitative studies were performed on an average of 12 anatomically matched whole cross-sections of brain and spinal cord as described earlier (Youssef *et al*., 2002).

siRNA transfections and treatments

For *in vitro* transfection of splenocytes, 2 µl TransIT-TKO transfection reagent (Mirus) was diluted in 50 µl serum-free/antibiotic-free Roswell Park Memorial Institute 1640 media per well. After 10 min incubation at room temperature, 1 µl 40 µM siRNA was added to 52 µl diluted transfection reagent. The siRNAs were incubated with the diluted transfection reagent at room temperature with gentle agitation for 30 min. The siRNAs were then added to the Vβ8.2 transgenic or B10.PL splenocyte cultures containing 5×10^6 cells in 500 µl media per well of a 24 well plate and incubated for 20 h at 37°C. On the following day, the cells were collected and washed with fresh media and then resuspended in 2 ml media and placed back in their original wells. MBP_{Ac1-11} peptide was added at 2 µg/ml. For Vα2.3/Vβ8.2 transgenic splenocytes, 2×10^6 splenocytes were placed in each well of a 24 well plate. The transfection protocol was the same, except the cells were placed with wild-type splenocytes (6×10^6 cells/well) that had been irradiated and cultured with MBP_{Ac1-11} after the 24 h transfection.

For *in vivo* experiments, 50 µg of *Prnp*-siRNA or nonsense-siRNA were administered intravenously (i.v.) at the time of immunization or the time of disease onset in 100 µl PBS via tail vein injection.

Immunofluorescence

Naïve mice, or mice that had been actively immunized for EAE, were injected intravenously with Cy3-labelled or DY547-labelled siRNAs. To visualize the fluorochrome-labelled siRNA *in situ*, tissues were prepared as described earlier (Puttaparthi *et al*., 2003). Briefly, the mice were sacrificed by injecting them with a lethal dose of sodium pentobarbital (250 mg/kg, i.p.) 24 or 72 h after injection of the siRNA. Anaesthetized mice were perfused transcardially with ice-cold PBS. Spinal cord, brain, liver and kidneys were removed and sectioned transversely at 250 µm intervals. Slices were transferred to Gey's balanced salt solution at room temperature. The slices were transferred onto slides and mounted with Gel/Mount. Slides were viewed with an E800 fluorescent microscope (Nikon Instruments Inc.) using Metamorph software.

Tracking of siRNA-labelled CD4 + T cells *in vivo*

Single cell suspensions of splenocytes obtained from Vα2.3Vß8.2 TCR transgenic mice were transfected with DY547-labelled *Prnp*-siRNA or nonsense-siRNA as described in the previous section. After 72 h of *in vitro* stimulation with MBP_{Ac1-11} (6 µg/ml) and IL-12 (0.5 ng/ml) the cells were collected, washed and resuspended at 5×10^6/200 µl. Each mouse ($n = 4$/siRNA) received 200 µl of siRNA transfected cells via i.p. injection. Seventy-two hours post injection, mice were euthanized and perfused with cold PBS. Numerous tissues, including the lymph nodes, spleen, lung, liver, brain and spinal cord, were collected, processed and examined for expression of DY547-labelled siRNA by flow cytometry (as described below) and immunofluorescence microscopy (described above).

CD4 T cell purification

Mouse $CD4^+$ T cells were purified from a bulk spleen population using a mouse CD4 T lymphocyte enrichment set (BD IMag^{TM}). The purity of $CD4^+$ T cells was assessed by flow cytometry and exceeded 95%.

Proliferation assays

For primary proliferation assays, splenocytes or lymph node cells were isolated from SJL/J mice that had been immunized with $PLP_{p139-151}$ seven days before, and that had been concomitantly treated with *Prnp*-siRNA or nonsense-siRNA. Cells were cultured with antigen in 96 well microtitre plates at a concentration of 5×10^6 cells/ml. Culture medium consisted of Roswell Park Memorial Institute medium 1640 (Invitrogen) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (5×10^{-5} M) and 1% (v/v) autologous normal mouse serum. Splenocytes were incubated for 72 h. Cultures were then pulsed for 18 h with 1 µCi per well of [^{3}H]thymidine before harvesting. Results are shown as mean of triplicates ± SEM.

For proliferation assays in which irradiated B10.PL splenocytes served as the antigen presenting cell, and MBP_{Ac1-11} TCR transgenic $CD4^+$-enriched T cells served as effector cells, splenocytes from B10.PL and MBP_{Ac1-11} TCR transgenic mice were brought into single cell suspension, and transfected with *Prnp*-siRNA or nonsense-siRNA. In other experiments, cells were treated for 24 h with the anti-mouse PrP^c monoclonal antibodies 19C4 or W226, or an anti-PrP^c single-chain variable fragment and the appropriate isotype control mouse IgG_1. Cells were then washed, irradiated and pulsed with MBP_{Ac1-11}. Cells were harvested on glass filters using a Tomtec harvester (Tomtec), and incorporated [*methyl*-^{3}H]thymidine was measured with a Betaplate counter (PerkinElmer).

Cytokine analysis

Supernatants from splenocytes cultured in parallel with those cells used in proliferation assays were used for cytokine analysis. Supernatants were collected for interleukin (IL)-2 (48 h), interferon gamma (IFNγ) and IL-17 (72 h), IL-4, IL-5 and IL-10 (120 h). Quantitative enzyme-linked immunosorbent assay (ELISA) was performed using paired monoclonal antibodies specific for corresponding cytokines as per manufacturer's recommendations (BD PharMingen). The results of ELISA experiments are expressed as an average of triplicate wells ± SD. A SOFTmax ELISA plate reader and software was used for data analysis (Molecular Devices).

Western blotting

Splenocytes were transfected as described above. For preparation of total cell lysates, cells were collected, spun down and resuspended in sodium dodecyl sulfate-lysis buffer. Cells were lysed on ice for 30 min and spun down to remove cell debris. Total cell lysates were also made from the brains of mice using a tissue homogenizer and lysing with sodium dodecyl sulfate-lysis buffer. Protease inhibitors (aprotinin, leupeptin and pepstatin) were added to all lysates at the time of preparation. Prior to the western blot, the protein concentration of these extracts was determined by using the BioRad Protein Assay. The extracts were diluted in sodium dodecyl sulfate loading buffer, boiled for 3 min and were electrophorectically separated on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. After transfer of the gels to nitrocellulose membranes, the membranes were blocked with 5% milk in tris-buffered saline. The primary antibody was diluted 1:1000 in blocking buffer and was added to the membrane for 2 h. The membrane was washed three times in tris-buffered saline/Tween. The secondary antibody was diluted 1:1000 and was added to the membrane for 1 h. The membrane was washed three times and a chemiluminescent substrate (Bio-rad Inc.) was added for 1 min; the blot was then exposed to film for various times (0.05–10 min). The density of the bands was determined by using ImageQuant 5.2 program. Data were normalized by dividing the density of the PrP^c band by the density of the β-actin band. The data are representative of at least five independent experiments. It is difficult to normalize between experiments, therefore, no error bars are shown.

Flow cytometry

The following antibodies were used: mouse anti-mouse PrP antibody (W226), fluorescein isothiocyanate-conjugated mouse IgG1 (BD Biosciences) and antigen presenting cell-conjugated anti-CD3 (eBioscience). Mouse spleens were pressed through a 70 μm nylon mesh cell strainer. Then, splenocytes were treated with RBC lysing buffer (Sigma-Aldrich). For flow cytometry, cells were counted, washed and resuspended in staining buffer (4% foetal calf serum and 0.1% sodium azide in PBS). Fc receptors were blocked with anti-CD16/32 (BD Biosciences) and the cells were incubated with 1 μg W226 monoclonal antibody for 45 min at 4°C. W226 staining was revealed with fluorescein isothiocyanate conjugated rat anti-mouse IgG1 followed by a 30 min blocking step with purified mouse immunoglobulins. After washing twice with staining buffer, cells were stained with directly labelled anti-CD3 for 30 min on ice, washed and then fixed with 1% paraformaldehyde. Twenty thousand gated events were acquired on a FACSCalibur (BD Biosciences) and analysed using FlowJo software (Tree Star).

Phosphorylation assay

$CD4^+$ T cells were activated by suspending cells in media with biotinylated anti-CD3 antibody (5 μg/ml) for 20 min, goat anti-hamster IgG (10 μg/ml Jackson Immunoresearch) for 1 min and then treated with anti-CD28 for 1 and 5 min, respectively. Sodium orthovanadate was then added to stop the phosphorylation and western blot analysis was performed.

T cells activation assays

A fast-growing variant of the D10 T cell clone (Kaye *et al*., 1983) was obtained from M. Krummel (University of California, San Francisco) and cultured as described (Kane *et al*., 2004). D10 cells (15 million) were transfected by electroporation as described (de Souza *et al*., 2005). For luciferase assays, cells were resuspended 18–20 h later at 2 million/ml in fresh media and stimulated for 6 h with biotinylated anti-CD3 and anti-CD28 antibodies (1 μg/ml each; BD Biosciences) plus streptavidin (5 μg/ml; Zymed) or phorbol 12-myristate 13-acetate (PMA) (25 ng/ml; Calbiochem) plus ionomycin (1 μm; Sigma). Luciferase activity was then measured as described (Kane *et al*., 2001). For determination of activation markers, cells were re-suspended the day after transfection at 1 million/ml and stimulated for 18 h with either media alone or anti-CD3/CD28 antibodies at 1 μg/ml each. Cells were then stained with phycoerythrin-conjugated antibodies to murine CD25 or CD69 (Caltag) and analysed on a BD LSR II flow cytometer.

Statistical analysis

Correlations between continuous and categorical variables were assessed using the Mann–Whitney U-test. The means of two normally distributed samples were compared by Student's *t*-test. All other statistical comparisons between groups were examined using one-way multiple range ANOVA test for multiple comparison. *P*-values <0.05 were considered significant.

Results

Peripheral silencing of PrP^C with siRNA does not affect central nervous system PrP^C expression

Prnp-specific siRNA and control NS-siRNA were generated to investigate the tissue specific role of PrP^c in activating and perpetuating cellular immune responses. By western blot analyses, we demonstrated that *in vitro* transfection of murine splenocytes with *Prnp*-siRNA substantially decreased PrP^c expression (Fig. 1A). On Day 3 after *in vivo* intravenous treatment of naïve SJL/J mice (Fig. 1B), and SJL/J mice immunized for EAE with 50 μg of *Prnp*-siRNA (Fig. 1C), PrP^c expression was decreased in splenocytes, but not in the brain (Fig. 1D). When evaluated by flow cytometry, PrP^c expression was found to be decreased by ~70% in $CD3^+$ T cells from *Prnp*-siRNA-treated mouse lymph node cells and splenocytes compared with nonsense-siRNA-treated samples (Fig. 1E). To investigate the bioavailability of

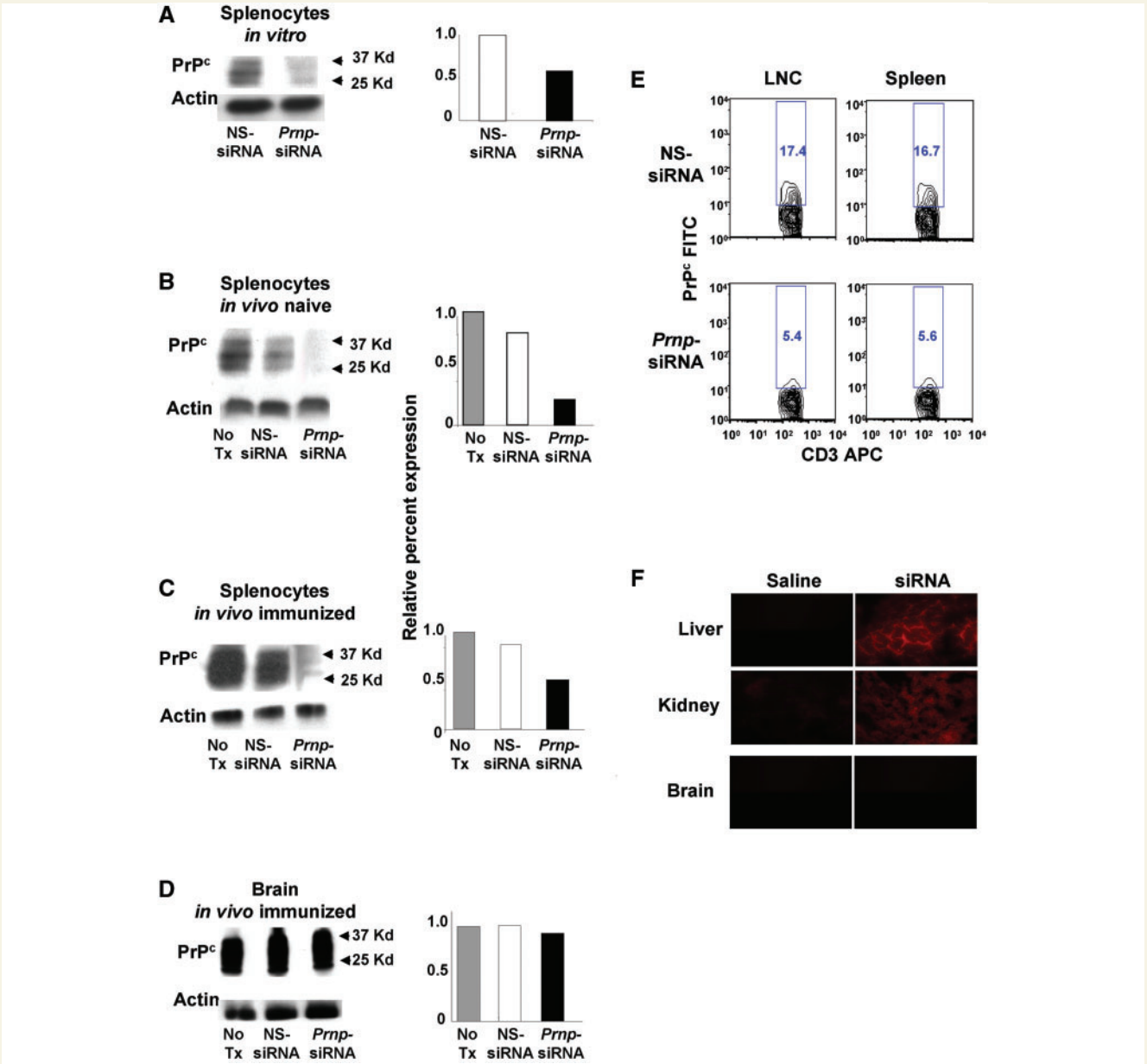

Figure 1 Decreased PrP^c expression levels on peripheral leucocytes but not in the CNS in mice treated with *Prnp*-siRNA. (**A**) Transfection with *Prnp*-siRNA decreased PrP^c expression in murine splenocytes, compared with nonsense (NS)-siRNA treatment. On Day 3 after *in vivo* treatment of (**B**) unimmunized SJL/J mice and (**C**) SJL/J mice immunized with $PLP_{p139-151}$ with 50 μg of *Prnp*-siRNA, PrP^c expression was decreased in splenocytes, but not in (**D**) brain homogenate of immunized mice. (**A–D**) Densitometry was performed, and relative PrP^c expression was determined by normalizing the PrP^c to β-actin levels. (**E**) Using flow cytometry three days after immunization and siRNA treatment, lymph node cells (LNC) and splenocytes were gated on CD3 and PrP^c. The top two panels show the percentage of PrP^{c+} cells within the CD3$^+$ cell population from nonsense-siRNA-treated murine cells. The lower two panels show the percentage of PrP^{c+} cells within the CD3$^+$ cell population from *Prnp*-siRNA-treated murine cells. PrP^c expression was decreased by ~70% following *Prnp*-siRNA treatment. (**F**) After injecting Cy3-labelled siRNA or DY547-labelled siRNA intravenously, fluorescence was detectable 72 h later in liver and kidney tissue but not in the brain of naïve mice or mice in which EAE had been induced by active immunization (data not shown).

Prnp-siRNA further, Cy3-labelled siRNA or DY547-labelled siRNA was injected intravenously into naïve experimental animals, or into mice in which EAE had been induced by active immunization. Fluorescence was detectable in the tissue sections of liver and kidney 72 h after injection (Fig. 1F), but not in the brain and spinal cord of naïve mice (Fig. 1F) or mice with active EAE (data not shown). Thus, *Prnp*-siRNA treatment created chimeric mice that expressed normal levels of PrP^c in

the CNS, but substantially decreased levels of PrP^c in peripheral organs.

Silencing PrP^c with siRNA worsens clinical and neuropathological central nervous system autoimmune disease

To test the role of PrP^c in CNS autoimmune disease, EAE was induced by active immunization of SJL/J mice with $PLP_{p139-151}$. A single dose of 50 μg *Prnp*-siRNA or nonsense-siRNA was injected i.v. via the tail vein in 100 μl PBS according to published methods (Lovett-Racke *et al*., 2004). Experimental animals were monitored for 40 days. In a set of 'prevention' experiments, treatment with *Prnp*-siRNA resulted in significantly worse clinical EAE within the first 10 days after disease onset than treatment with nonsense-siRNA or no treatment (Fig. 2A, Supplementary Table S1A). In a second set of 'treatment' experiments, mice treated with *Prnp*-siRNA had a significantly worse initial clinical exacerbation than control mice and continued to do significantly worse throughout the observation period (Fig. 2B, Supplementary Table S1B)

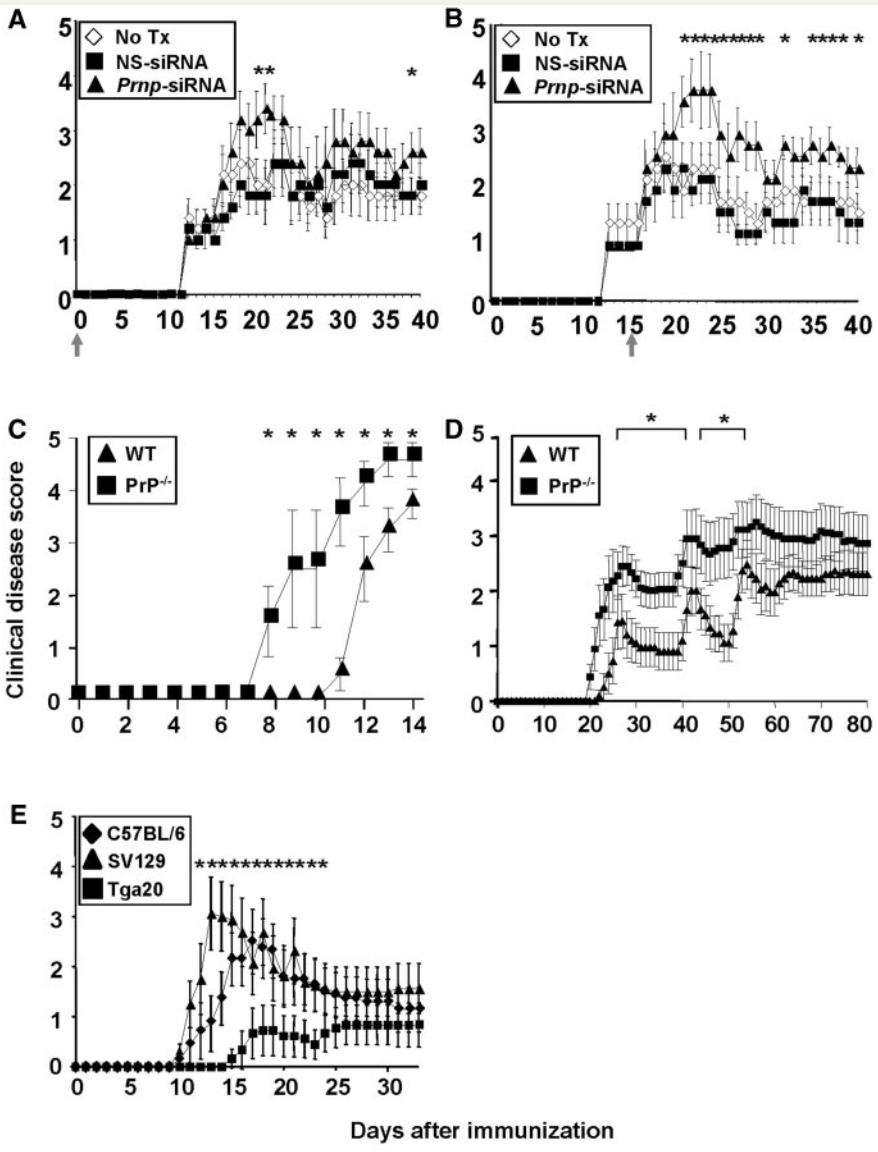

Figure 2 *Prnp*-siRNA treated and PrP-deficient ($PrP^{-/-}$) mice develop more severe EAE whereas mice overexpressing PrP^C are protected. (**A** and **B**) *In vivo* silencing of PrP^c worsens the clinical course of actively induced EAE in SJL/J mice. (**A**) In an EAE 'prevention' experiment, SJL/L mice were injected intravenously with a single dose of *Prnp*-siRNA or nonsense (NS)-siRNA at the time of immunization with $PLP_{p139-151}$, as indicated by a grey arrow. *Prnp*-siRNA treatment resulted in clinically more severe disease. (**B**) In an EAE 'treatment' experiment, *Prnp*-siRNA or nonsense-siRNA was injected at the time of clinical onset of EAE (grey arrow). Mice treated with *Prnp*-siRNA had a significantly worse initial clinical exacerbation. (**C**) Male and (**D**) female $PrP^{-/-}$ mice had earlier disease onset and significantly higher disease scores than male wild-type (WT) mice. (**E**) Tga20 mice had a delayed disease onset, and developed only mild EAE compared to C57BL/6 and Sv129 wild-type mice.

Histological evaluation of CNS tissue with haematoxylin and eosin (data not shown) revealed a significantly increased number of inflammatory foci in the brain ($P = 0.046$; data not shown) and spinal cord parenchyma ($P = 0.04$; Supplementary Fig. S1D) in EAE mice treated with *Prnp*-siRNA. In the brain, the difference in inflammatory infiltrates was also significant within the meninges ($P = 0.008$; data not shown).

Next, we performed active immunization to induce EAE in PrP$^{-/-}$ mice (Manson *et al*., 1994) on the Sv129/OLA background (H-2b), and in Sv129/OLA wild-type mice. Male PrP$^{-/-}$ mice had earlier disease onset and significantly higher disease scores than male wild-type mice (Fig. 2C, Supplementary Table S1C). Similarly, female PrP$^{-/-}$ mice had earlier disease onset and worse clinical disease than female wild-type mice over a longer period of 80 days (Fig. 2D, Supplementary Table S1D). Histopathology obtained at Day 29 after disease induction revealed significantly higher numbers of inflammatory infiltrates in PrP$^{-/-}$ mice compared with wild-type controls (Supplementary Fig. S1A–C).

To determine whether the increased number of inflammatory cells in the CNS of mice treated with *Prnp*-siRNA are antigen-specific T cells in which *Prnp* was silenced, we tracked Vα2.3Vß8.2 TCR transgenic CD4$^+$ T cells transfected with DY547-labelled *Prnp*-siRNA or nonsense-siRNA *in vivo*. Numerous tissues, including the lymph nodes, spleen, lung, liver, brain and spinal cord were collected, processed and examined for expression of DY547-labelled siRNA by flow cytometry and immunofluorescence microscopy. With regard to absolute cell numbers and percent of fluorochrome-labelled CD4$^+$ T cells there was no difference in any of the organs between cells in which *Prnp* had been silenced, and in those in which it had not been silenced by siRNA (data not shown).

Mice overexpressing PrP^c are protected from severe experimental autoimmune encephalomyelitis

Tga20 mice that ubiquitously overexpress PrP^c (Fischer *et al*., 1996) had a later disease onset and developed milder disease than C57BL/6 wild-type mice and Sv129 wild-type mice that were used as controls (Fig. 2E, Supplementary Table S1E). Consistent with published data (Zabel *et al*., 2009), we observed a significantly lower absolute number of CD4$^+$ and CD8$^+$ T cells in the spleen of Tga20 mice compared with wild-type controls (data not shown). Other investigators had previously demonstrated that mRNA transcripts from pre-TCR-α, a T-cell development gene located on mouse chromosome 17, are substantially reduced in Tga20 mice (Zabel *et al*., 2009). Interestingly, the percentage of FoxP3$^+$ T regulatory T cells was increased in these mice compared with wild-type controls (data not shown).

Silencing PrP^c on antigen-specific T cells promotes the differentiation into Th1 and Th17 phenotypes

Splenocytes from SJL/J mice immunized with $PLP_{p139–151}$ and treated intravenously with 50 μg of *Prnp*-siRNA or nonsense-siRNA were brought into single cell suspension 3 days after immunization and were pulsed with $PLP_{p139–151}$. IL-2, IFNγ, IL-17, IL-4, IL-5 and IL-10 cytokine expression in culture supernatants was measured by ELISA. Treatment of mice with *Prnp*-siRNA significantly increased the expression of IL-2 (Fig. 3A), interferon-γ (Fig. 3B) and IL-17 (Fig. 3C). In contrast, there was no effect of silencing PrP^c with siRNA on the expression of IL-4 (Fig. 3D), IL-5 (Fig. 3E) and IL-10 (Fig. 3F). Protein expression of T box expressed in T cells, a transcription factor that regulates IFNγ and IL-17 expression, and (H) retinoic acid receptor-related orphan receptor (ROR)γt, a regulator of IL-17 was increased in splenocytes of mice treated with *Prnp*-siRNA compared with nonsense-siRNA-treated cells (Fig. 3G and H). The expression of the Th2 transcription factor GATA binding protein (GATA)-3 was not altered (Fig. 3I).

Silencing of PrP^c augments antigen-specific T cell proliferation and activation

Lmph node cells (Fig. 4A) and splenocytes (Fig. 4B) from *Prnp*-siRNA-treated mice showed significantly increased antigen-specific proliferation compared with cells from nonsense-siRNA-treated control mice. In an effort to understand the function of PrP^c in T cells further, we tested the activation status of the *Prnp*-siRNA-treated T cells. As shown in Fig. 4C, silencing of PrP^c augmented the expression of the activation marker CD25 in T cells stimulated with anti-CD3, with or without addition of anti-CD28 monoclonal antibody. The effect on CD25 appeared to be most pronounced in the presence of both anti-CD3 and anti-CD28. There was less of an effect of *Prnp*-siRNA treatment on the expression of CD69, an early T cell activation marker (Fig. 4D) (Testi *et al*., 1989), although some increase in CD69 expression was observed in conjunction with anti-CD3 stimulation alone.

T cells, not antigen presenting cells, mediate the proinflammatory effects of PrP^C silencing

PrP^c is constitutively expressed in T cells (Cashman *et al*., 1990; Durig *et al*., 2000; Li *et al*., 2001) and cells that can serve as antigen-presenting cells, including B cells and dendritic cells. Thus, we investigated whether the effect of PrP^c on T cell proliferation was predominantly mediated through its effects on T cells, or antigen-presenting cells or both. Specifically, sublethally irradiated splenocytes from B10.PL mice were used as antigen-presenting cells and presented MBP peptide Ac1–11 to CD4$^+$-enriched $MBP_{1–11}$ TCR transgenic T cells (Goverman *et al*., 1993). Either antigen-presenting cells or T cells were treated with *Prnp*-siRNA (Fig. 5A), a recombinant anti-PrP^c single chain variable fragment (Fig. 5B), or two distinct anti-PrP^c monoclonal antibodies (Fig. 5C and D) at a dose of 1 μg/ml for 24 h prior to the proliferation assay (for details, see Experimental Procedures section). Silencing of PrP^c in antigen-specific T cells resulted in increased proliferation of these cells (Fig. 5A–D). In contrast,

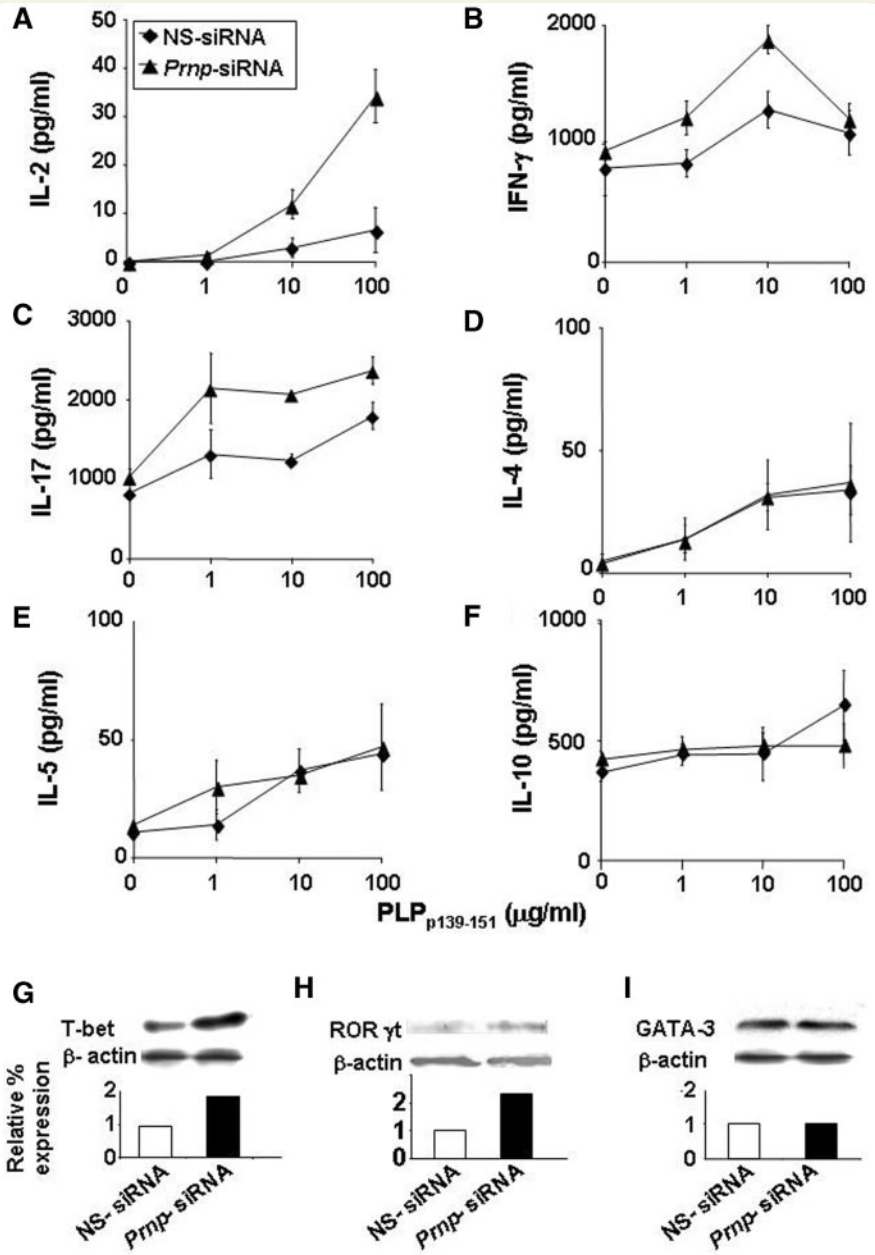

Figure 3 Silencing PrP^c in antigen specific splenocytes increases the expression of Th1 cytokines by upregulating T box expressed in T cells (T-bet). Splenocytes from SJL/J mice immunized with $PLP_{p139–151}$ and treated concomitantly intravenously with 50 μg of *Prnp*-siRNA or nonsense (NS)-siRNA, were brought into single cell suspension 3 days after immunization and were pulsed with $PLP_{p139–151}$. Interleukin (IL)-2, interferon (IFN)-γ, IL-17, IL-4, IL-5 and IL-10 cytokine expression was measured by ELISA. Silencing of PrP^c significantly increased the expression of (**A**) IL-2, (**B**) IFN-γ and (**C**) IL-17. In contrast, silencing of PrP^c had no effect on the expression of (**D**) IL-4, (**E**) IL-5 and (**F**) IL-10. (**G**) Protein expression of T box expressed in T cells and (**H**) retinoic acid receptor related orphan receptor (ROR)γt in splenocytes of mice treated with *Prnp*-siRNA was increased compared with nonsense-siRNA-treated cells. (**I**) In contrast, the expression of GATA binding protein (GATA)-3 was not altered. The data are shown as mean clinical score ± standard deviation.

silencing of PrP^c in antigen-presenting cells had no apparent effect on antigen-specific T cell proliferation (Fig. 5A–D).

To test the *in vivo* relevance of this observation, reciprocal adoptive transfer EAE experiments were conducted. $PLP_{p139–151}$-specific T cells that were treated with *Prnp*-siRNA or nonsense-siRNA were adoptively transferred into recipient mice treated with either *Prnp*-siRNA or nonsense-siRNA. Significant worsening of clinical EAE was observed in (i) recipient mice treated with *Prnp*-siRNA that received untreated donor cells; (ii) recipient mice treated with *Prnp*-siRNA that received *Prnp*-siRNA-treated donor cells and (iii) recipient mice treated with nonsense-siRNA that received *Prnp*-siRNA-treated donor cells (Fig. 5E,

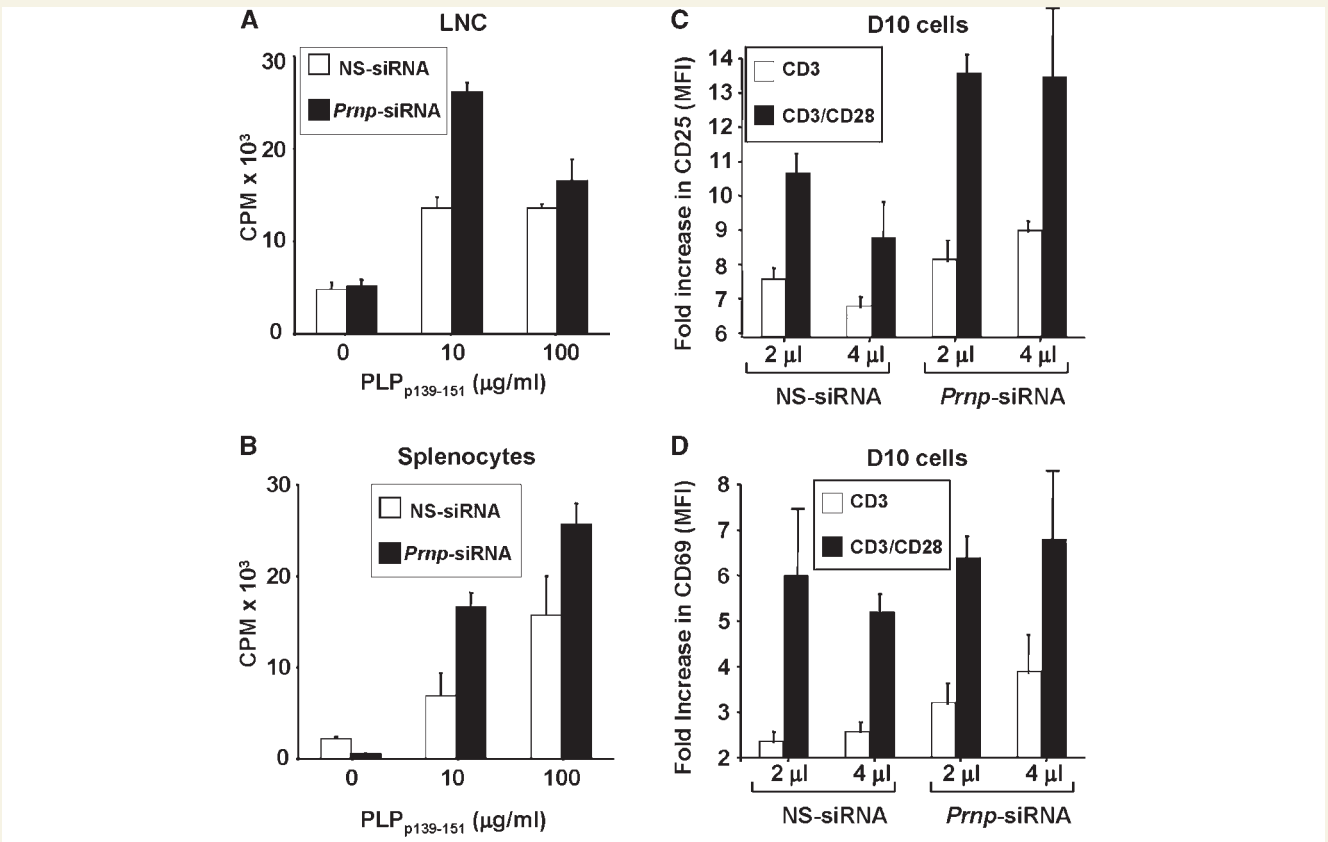

Figure 4 Silencing PrP^c in antigen-specific T cells increases T cell proliferation and activation markers. (**A**) Lymph node cells (LNC) and (**B**) splenocytes from SJL/J mice immunized with $PLP_{p139–151}$ and treated with *Prnp*-siRNA or nonsense (NS)-RNA at the time of immunization were brought into single cell suspension on day 10 after immunization. Both (**A**) Lymph node cells and (**B**) splenocytes from *Prnp*-siRNA-treated mice showed increased proliferation. (**C**) *Prnp*-siRNA treatment also significantly increased the mean fluorescence intensity (MFI) of the activation marker CD25 in D10 T cells activated by anti-CD3, with or without anti-CD28 monoclonal antibody. (**D**) There was less of an effect of *Prnp*-siRNA treatment on the expression of CD69, an early T cell activation marker. Proliferation was measured as counts per minute (CPM).

Supplementary Table S1F). PrP^c expression in draining lymph nodes was diminished in lymph node cells until Day 6 post siRNA administration (Fig. 5F).

Silencing of PrP^c in anti-CD3/CD28 activated T cells increases zeta-chain-associated protein-70 phosphorylation and nuclear factor of activated T cells/activator protein 1 reporter activity

Our earlier results on the effects of PrP^c on antigen-specific T cell activation, proliferation and differentiation suggested that PrP^c may serve as an immunomodulatory molecule and specifically that the absence of PrP^c amplified TCR signalling. Zeta-chain-associated protein (ZAP)-70, a member of the protein tyrosine kinase family, is constitutively expressed in T lymphocytes. The phosphorylation of ZAP-70 and its association with the zeta chain are upstream events in the TCR signalling pathway. *Prnp*-siRNA, but not nonsense-siRNA, treatment was associated with increased phosphorylation of ZAP-70 and the zeta chain of the TCR/CD3 complex (Fig. 6A). There was a less pronounced but detectable increase in tyrosine phosphorylation of the ZAP-70 substrate linker of activated T cells (Fig. 6A).

After activation with anti-CD3 and anti-CD28 monoclonal antibodies, transcription of an nuclear factor of activated T cells (NFAT)/activator protein 1 (AP-1) luciferase reporter was significantly increased in T cells treated with *Prnp*-siRNA compared with nonsense-siRNA (Fig. 6B). There was also a significant increase in unstimulated T cells. In contrast, NFAT/AP-1 reporter activity was not altered in phorbol 12-myristate 13-acetate and ionomycin stimulated T cells (Fig. 6B), consistent with the notion that the effects of PrP^c silencing were primarily at the level of TCR-proximal signalling.

Silencing PrP^c induces spontaneous experimental autoimmune encephalomyelitis in $MBP_{1–11}$ T cell receptor transgenic mice

We treated two distinct $MBP_{1–11}$ TCR transgenic mice generated by Goverman *et al*. (1993) and Lafaille *et al*. (1994) with *Prnp*-

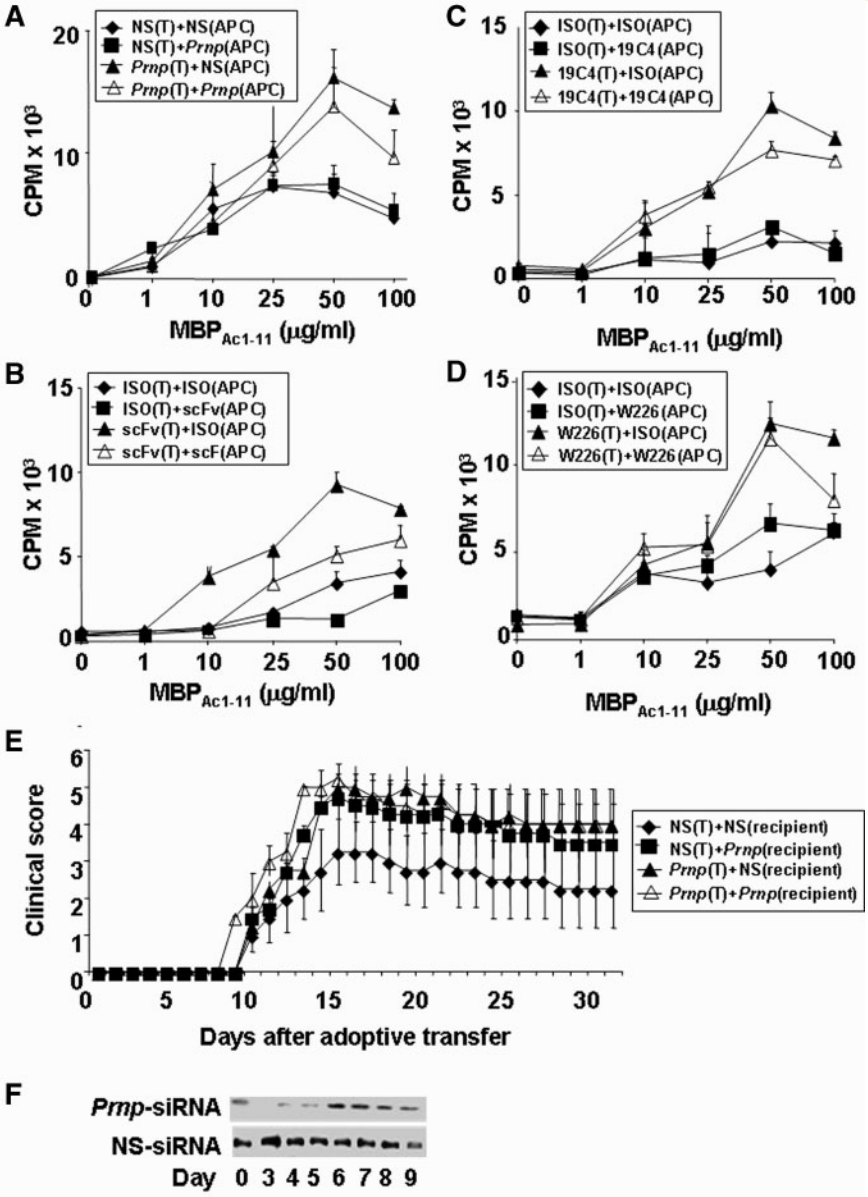

Figure 5 Antigen-specific T cells are the main target of *Prnp*-siRNA. (**A**) Irradiated splenocytes from B10PL mice that served as antigen presenting cells (APC), or $MBP_{1–11}$ TCR transgenic $CD4^+$ T cells were treated with *Prnp*-siRNA and/or nonsense-siRNA, and incubated with antigen. Silencing of PrP^c in T cells results in increased proliferation, whereas silencing of PrP^c in antigen presenting cells had no apparent effect on T cell proliferation. Targeting PrP^c in T cells with a (**B**) single chain variable fragment (scFv), or two monoclonal mouse anti-mouse PrP IgG_1 monoclonal antibodies (**C**) 19C4, (**D**) W226 also resulted in the augmented proliferation of antigen-specific T cells. (**E**) Mice treated with *Prnp*-siRNA as recipients of *Prnp*-siRNA-treated adoptively transferred T cells, as recipients of direct *Prnp*-siRNA treatment, or both, developed significantly worse EAE than controls. (**F**) PrP^c expression after *Prnp*-siRNA treatment is suppressed for 5 days.

siRNA. $MBP_{1–11}$ TCR transgenic mice on a B10.PL background ($I\text{-}A^u$) generated by Goverman and coworkers (1993) were reported to have a higher incidence of spontaneous EAE in non-pathogen-free conditions than the mice created by Lafaille *et al*. (1994). Without the addition of antigen, only mice generated by Goverman *et al*. rapidly developed spontaneous EAE after treatment with *Prnp*-siRNA on Days 0, 3 and 6 of the observation period (indicated by red arrows), but not after treatment with nonsense-siRNA (Fig. 6C).

Silencing PrP^c promotes T cell survival

To investigate whether an effect of PrP^c on the survival of activated antigen-specific T cells may account for some of its effects on CNS autoimmune disease, *Prnp*-siRNA or nonsense-siRNA-transfected purified $OVA_{323–339}$ TCR transgenic mice $CD45.2^+$ $CD4^+$ T cells were transferred intravenously into C57BL/6 $CD45.1^+$ wild-type mice that were immunized with $OVA_{323–339}$ in complete freund's adjuvant 24 h after cell transfer.

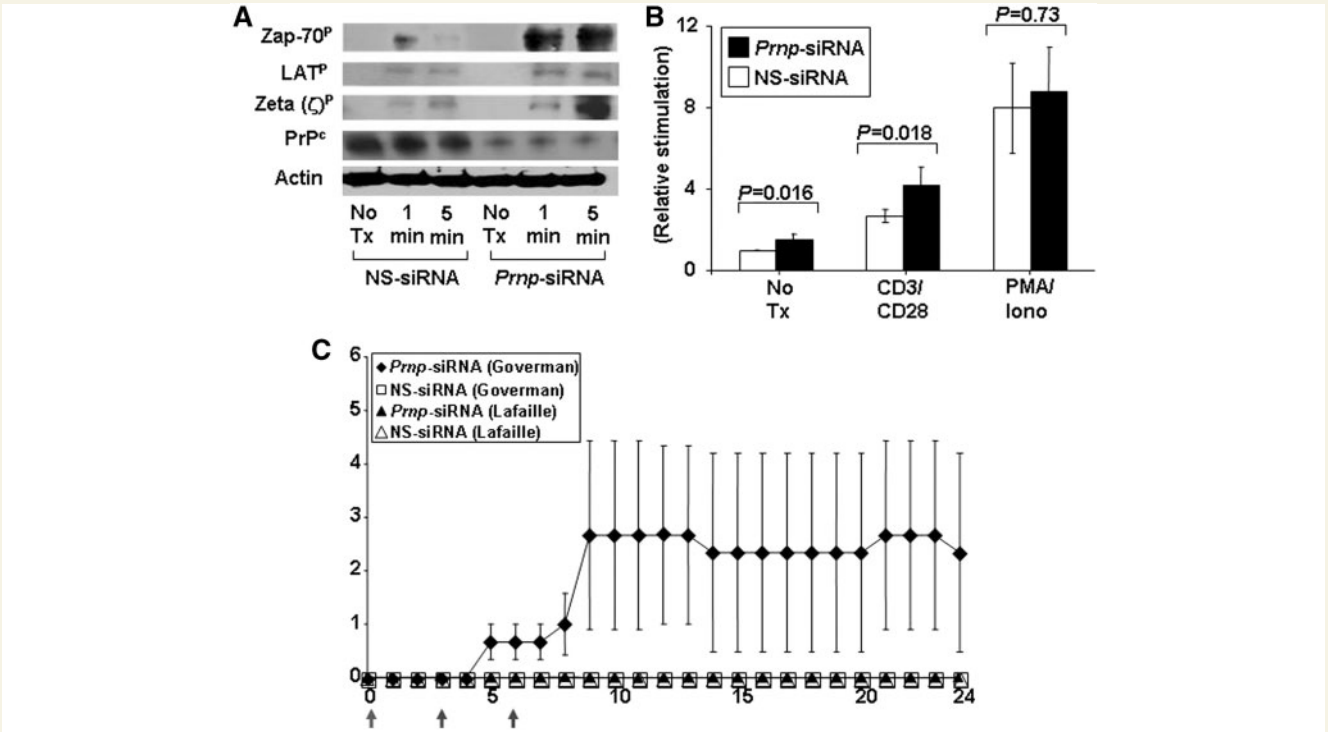

Figure 6 Silencing PrP^c on T cells increases ZAP-70 phosphorylation and NFAT/AP-1 reporter activity. (**A**) Phosphorylation of ZAP-70 and the zeta chain of the TCR/CD3 complex was increased in activated CD4$^+$ T cells treated with *Prnp*-siRNA. (**B**) Transcription of an NFAT/AP-1 luciferase reporter was also significantly increased in activated and unstimulated T cells treated with *Prnp*-siRNA. In contrast, NFAT/AP-1 reporter activity was not altered in PMA/ionomycin-stimulated T cells. (**C**) MBP peptide AC1–11 TCR transgenic mice on a B10.PL background (I-A^u) generated by Joan Goverman and coworkers developed EAE after *in vivo* intravenous treatment with *Prnp*-siRNA on Days 0, 3 and 6 of the observation period (indicated by grey arrows), but not after treatment with nonsense-siRNA. In contrast, MBP$_{1–11}$ TCR transgenic mice generated by Lafaille and coworkers did not develop disease.

We were able to recover a significantly higher number of *Prnp*-siRNA-transfected CD45.2$^+$ donor OVA$_{323–339}$ TCR transgenic CD4$^+$ T cells from lymph nodes and spleens than nonsense-siRNA-transfected cells (Fig. 7A). At Day 5 post transfer, there was no difference with regard to *in vivo* antigen-specific proliferation between OVA$_{323–339}$ TCR transgenic CD4$^+$ T cells transfected with *Prnp*-siRNA (Fig. 7B), or with nonsense-siRNA (Fig. 7C) as shown by flow cytometric proliferation analysis of 5- (and 6-) carboxy fluorescein diacetate succinimidyl ester-labelled OVA$_{323–339}$ TCR transgenic CD45.2$^+$ CD4$^+$ T cells. When lymph nodes (Fig. 7D) and splenocytes (Fig. 7E) of recipient mice were resected on Day 3 after immunization, and recall assays were performed with OVA$_{323–339}$, we observed a significant increase in cell proliferation in both compartments. Thus, while there was no change in proliferative responses in antigen-specific T cells in both secondary lymphoid organs, there appeared to be bystander-activation of other cells.

Next, purified CD4$^+$ MBP$_{1–11}$ TCR transgenic T cells from Lafaille *et al*. (1994) MBP$_{1–11}$ TCR transgenic mice were activated *in vitro* with 2 μg/ml MBP$_{1–11}$ for 72 h. The number of nonsense-siRNA-transfected T cells started to decline on Day 3 after putting the cells into culture, and none of the cells in this experimental group were viable on Day 10 (Fig. 7F). In contrast, cell numbers remained relatively stable in the CD4$^+$ MBP$_{1–11}$ TCR transgenic T cells transfected with *Prnp*-siRNA (Fig. 7F).

Discussion

Although it is well-known as a substrate for PrP^{Sc} in prion diseases, the normal functions of PrP^c in cellular processes and non-prion CNS diseases remain enigmatic. In the EAE model of CNS autoimmunity, we describe that PrP^c is an important inhibitor of peripheral immune function. Deletion, knockdown or blockade of PrP^c resulted in accelerated and more severe autoimmunity while PrP^c overexpression (on a PrP knockout background) resulted in diminished autoimmunity.

Consistent with recently published findings (Tsutsui *et al*., 2008; Ingram *et al*., 2009), we have found that EAE severity was significantly increased in PrP knockout mice. Based purely on the knockout results, however, it remained unclear whether the effects of PrP^c on CNS autoimmunity were due to alterations in the susceptibility of the CNS to inflammation (i.e. neuronal survival) or due to alterations of the inflammatory response. Additionally, because the knockout animals lack all PrP^c expression, it was not apparent whether alterations in immune function were due to defects in development or thymic negative selection. Pharmacological *in vivo* silencing of PrP^c using *Prnp*-siRNA allowed us to define the role of this molecule more precisely in different disease stages. PrP^c expression was effectively silenced solely in cells of the peripheral immune system but not brain tissue

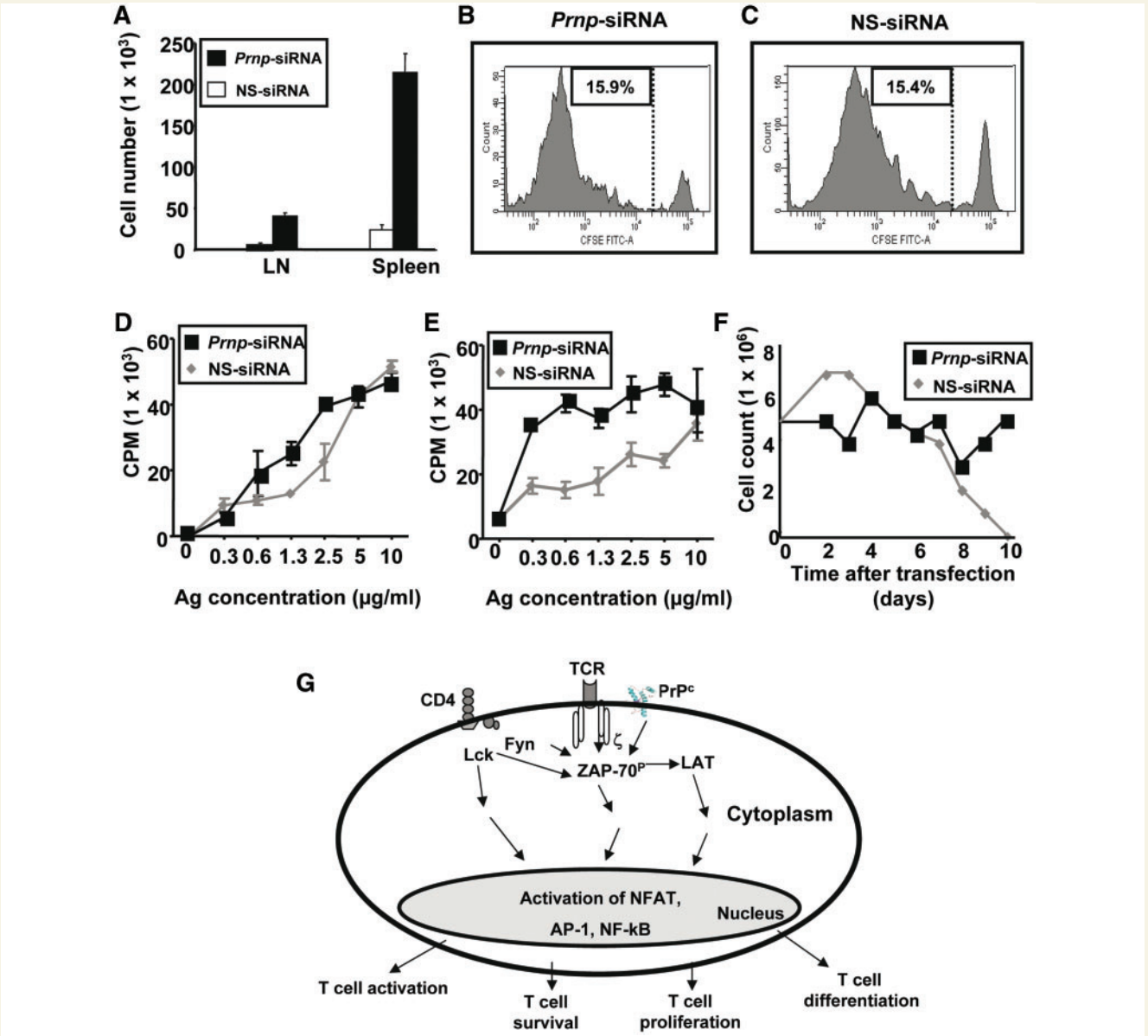

Figure 7 Silencing of PrP^c improves T cell survival. *Prnp*-siRNA or nonsense-siRNA-transfected purified $OVA_{323–339}$ TCR transgenic $CD45.2^+$ $CD4^+$ T cells were transferred intravenously into C57BL/6 $CD45.1^+$ wild-type mice that were immunized with $OVA_{323–339}$ in complete freund's adjuvant 24 h after cell transfer. (**A**) A significantly higher number of *Prnp*-siRNA-transfected $CD45.2^+$ donor $OVA_{323–339}$ TCR transgenic $CD4^+$ T cells were recovered from lymph nodes and spleens than nonsense-siRNA-transfected cells. At Day 5 post transfer, there was no difference with regard to *in vivo* antigen-specific proliferation between $OVA_{323–339}$ TCR transgenic $CD4^+$ T cells transfected with (**B**) *Prnp*-siRNA, or with (**C**) nonsense-siRNA. When (**D**) lymph nodes and (**E**) splenocytes of recipient mice were resected on Day 3 after immunization, and recall assays were performed with $OVA_{323–339}$, a significant increase in cell proliferation in both compartments was observed. (**F**) The number of purified antigen-activated $CD4^+$ $MBP_{1–11}$ TCR transgenic T cells transfected with nonsense-siRNA started to decline *in vitro* on Day 3, and none of the cells were viable on Day 10. Cell numbers remained relatively stable in the $CD4^+$ $MBP_{1–11}$ TCR transgenic T cells treated with *Prnp*-siRNA. (**G**) Schematic of proposed mechanisms of action of PrP^c in antigen-specific T cells.

after treatment with *Prnp*-siRNA. In addition, Cy3-labelled siRNA was undetectable in CNS tissue indicating that the major location of siRNA knockdown is the periphery. Consequently, we conclude that the major effects of PrP^c in EAE pathogenesis are based on the decrease in PrP^c expression in lymphatic tissues. Furthermore, *Prnp*-siRNA administration in adult mice resulted in rapidly enhanced immune responses, thereby bypassing the potential for thymic defects to account for the observed exacerbated autoimmunity. Although we cannot discount the function of PrP^c in the thymic maturation processes in the knockout mouse, it appears that PrP^c disruption in the peripheral immune system is sufficient to enhance immune responses.

The finding that PrP^c-silencing could exacerbate disease when delivered at the time of immunization, onset of disease, or at later

stages of autoimmunity strongly suggests that PrP^c is an important negative regulator of T cell responses.

Our data on *in vivo* T cell tracking of cells treated with fluorescent *Prnp*-siRNA or nonsense-siRNA strongly suggest that some of the effects we observed in mice treated with *Prnp*-siRNA, including the increased number of inflammatory infiltrates in the CNS, may be an indirect effect of *Prnp*-silencing on other lymphocyte populations, resulting in an amplification of the initial immune response. These data are complementary to observations we made in our adoptive transfer experiments (Fig. 5E, Supplementary Table S1F) and in some of the survival experiments. Specifically, *Prnp*-siRNA or nonsense-siRNA-transfected purified $OVA_{323–339}$ TCR transgenic CD45.2$^+$ CD4$^+$ T cells were transferred intravenously into C57BL/6 CD45.1$^+$ wild-type mice that were immunized with $OVA_{323–339}$ in complete freund's adjuvant 24 h after cell transfer to investigate whether an effect of PrP^c on the survival of activated antigen-specific T cells may account for some of its effects on CNS autoimmune disease. At Day 5 post transfer, there was no difference with regard to *in vivo* antigen-specific proliferation between $OVA_{323–339}$ TCR transgenic CD4$^+$ T cells transfected with *Prnp*-siRNA (Fig. 7B), or with nonsense-siRNA (Fig. 7C). In contrast, silencing of *Prnp in vitro* resulted in increased proliferation of bulk T cell populations obtained from splenocytes (Fig. 5A–D).

To study the role of PrP^c as a negative regulator of T lymphocyte responses in more detail, we examined the effects of PrP^c knockdown on T cell activation and survival.

PrP^c is a negative regulator of TCR signalling

We consistently observed an increased antigen-specific proliferation when PrP^c was silenced only on T cells, rather than on T cells and antigen-presenting cells. At this point, we are investigating the differential effect of PrP^c silencing and overexpression on different cell types. From preliminary data it appears that silencing of PrP^c in dendritic cells may be pro-apoptotic, while it is anti-apoptotic in T cells.

TCR signalling is critical to the development of EAE at the immunization and elicitation phases of the disease. Among the earliest events of TCR engagement is recruitment of leukocyte-specific protein tyrosine kinase and subsequent phosphorylation of the TCR zeta chain. Phosphorylated zeta serves as a binding site for ZAP-70 (Chan *et al*., 1992), which then phosphorylates numerous substrates including linker of activated T cells. Although it is so far unclear how PrP^c interacts with or influences ZAP-70, PrP^c immunoprecipitated with ZAP-70 in activated T cells in a previous study (Mattei *et al*., 2004).

We found an increased phosphorylation of ZAP-70 in activated T cells after PrP^c silencing. Suppression of the ZAP-70 dependent pathway by PrP^c was confirmed by demonstrating that basal and CD3/CD28-stimulated transcription from NFAT/AP-1, two transcription factors crucial in T cell activation and differentiation, were also enhanced upon silencing PrP^c in T cells (Fig. 6B). Interestingly, NFAT transcription was not altered in the PMA/ionomycin-stimulated T cells. Due to the fact that PMA/ionomycin induces NFAT/AP-1-dependent transcription by activation of protein kinase C/Ras/mitogen-activated protein kinase and calcium mobilization, respectively (Chatila *et al*., 1989; Liu and Heckman 1998), our results support the notion that PrP^c functions as a regulator of TCR-proximal signalling. The *in vivo* importance of PrP^c as a negative regulator of TCR signals was demonstrated by the increased incidence of spontaneous EAE in the autoimmune-prone $MBP_{1–11}$ TCR-transgenic mice after treatment with *Prnp*-siRNA (Fig. 6C).

We also demonstrate that PrP^c may exert an indirect immunosuppressive function by promoting T cell death. Increased numbers of antigen-specific T cells *in vivo* after silencing of *Prnp* allow several explanations: (i) a decreased sequestration of these cells from secondary lymphoid organs into other compartments; (ii) a decrease of Fas-mediated apoptotic cell death; (iii) a diminished growth factor withdrawal due to decreased antigen-specific proliferation or (iv) a push of these cells into cell cycle. The latter scenario was basically ruled out by showing that there was no difference in proliferation of antigen-specific T cells transfected with *Prnp*-siRNA or nonsense-siRNA. Furthermore our results on increased T cell survival *in vitro* after silencing of PrP^c strongly argue against the hypothesis of diminished growth factor withdrawal from the *Prnp*-siRNA-treated cells, as more cells were competing for growth factors in a closed system for a 10 day period. These results also rule out differences in tissue sequestration between the two treatment groups. Thus, PrP^c may promote apoptosis in activated antigen-specific T cells, possibly mediated through a Fas pathway, or other tumor necrosis factor receptor superfamily member pathways.

Summary and proposed model for PrP^c

In summary, we reveal PrP^c as a molecule critically influencing T cell functions, by modulating TCR signals and limiting survival. We can thereby clearly assign an essential function to PrP^c beyond being the replicative substrate or transport molecule for prions. Whether the conversion from PrP^c to the infectious PrP^{Sc} conformer also involves changes in T cell function, and whether these have anything to do with prion disease mechanisms is currently unclear. It is possible that the effect for PrP^c on regulating TCR signals may suggest a similar function in other cell types. Other cellular receptors, including the B cell receptor, Fc receptor, epidermal growth factor receptor and insulin receptor possess a similar signalling architecture as the TCR. Our observations put PrP^c at the intersection of neuroinflammation and neurodegeneration.

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Supplementary material

Supplementary material is available at *Brain* online.

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