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Multiple sclerosis associated genetic variants of *CD226* impair regulatory T cell function

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Recent association studies have linked numerous genetic variants with an increased risk for multiple sclerosis, although their functional relevance remains largely unknown. Here we investigated phenotypical and functional consequences of a genetic variant in the *CD226* gene that, among other autoimmune diseases, predisposes to multiple sclerosis. Phenotypically, effector and regulatory $CD4^+$ memory T cells of healthy individuals carrying the predisposing *CD226* genetic variant showed, in comparison to carriers of the protective variant, reduced surface expression of CD226 and an impaired induction of CD226 after stimulation. This haplotype-dependent reduction in CD226 expression on memory T cells was abrogated in patients with multiple sclerosis, as CD226 expression was comparable to healthy risk haplotype carriers irrespective of genetic variant. Functionally, FOXP3-positive regulatory T cells from healthy carriers of the genetic protective variant showed superior suppressive capacity, which was again abrogated in multiple sclerosis patients. Mimicking the phenotype of human CD226 genetic risk variant carriers, regulatory T cells derived from *Cd226*-deficient mice showed similarly reduced inhibitory activity, eventually resulting in an exacerbated disease course of experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis. Therefore, by combining human and mouse analyses we show that CD226 exhibits an important role in the activation of regulatory T cells, with its genetically imposed dysregulation impairing regulatory T cell function.

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Abbreviations: EAE = experimental autoimmune encephalomyelitis; Treg = regulatory T cell

Introduction

Multiple sclerosis is the most frequent chronic demyelinating disease of the CNS, characterized by destruction of CNS tissue by infiltrating immune cells. It is believed that multiple sclerosis and other autoimmune diseases develop in genetically predisposed individuals exposed to certain environmental conditions. Several genetic association studies have identified a number of genes, which predispose for multiple sclerosis (Sawcer *et al.*, 2011). With few

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exceptions (Gregersen *et al.*, 2006; Friese *et al.*, 2008; Gregory *et al.*, 2012), the functional relevance of these variants remains largely unknown. Among the genetic risk factors a variant in the gene encoding the protein DNAX-accessory molecule 1 (DNAM-1; *CD226*) has been identified to predispose to type 1 diabetes (Todd *et al.*, 2007), rheumatoid arthritis (Okada *et al.*, 2013) and multiple sclerosis (Hafler *et al.*, 2009), among other autoimmune diseases (Qiu *et al.*, 2013). In the immune system CD226 is expressed on T cells and monocytes and has recently been identified as an important element in a T cell co-signalling pathway (Chan *et al.*, 2012). In this context, CD226 acts as a co-signalling activator, whereas T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) resembles the inhibitory counterpart, both binding the ligands CD112 and CD155 (encoded by *PVRL2* and *PVR*, respectively) (Yu *et al.*, 2009).

Initially, CD226 was described as a key molecule on the surface of Th1-polarized cells and that blockade of CD226 ameliorates the course of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (Dardalhon *et al.*, 2005). Later studies provided evidence that CD226 is in fact also highly expressed in Th17 cells, but absent in Th2 cells (Lozano *et al.*, 2013). In contrast to the co-stimulatory activity of CD226, TIGIT serves co-inhibitory functions and competes with CD226 for the shared ligand CD155 (Yu *et al.*, 2009). In the absence of TIGIT, as shown in *Tigit*-deficient mice, immunized mice suffer from an exacerbated disease course of EAE (Joller *et al.*, 2011). Furthermore, it has been shown that TIGIT is expressed on a subset of regulatory T cells (Treg), which selectively inhibit Th1 and Th17 cell responses (Joller *et al.*, 2014). Recently, CD226 has been identified to be expressed in IL10-producing FOXP3-negative $CD4^+$ type 1 T regulatory (T_R1) cells (Gagliani *et al.*, 2013) and in classical $FOXP3^+$ regulatory T cells (Koyama *et al.*, 2013), although its precise function in the context of immune regulation remains unknown.

Besides effector cells, $CD4^+FOXP3^+$ Treg cells depend on co-signalling molecules to exert their full immunosuppressive capacity and prevent autoimmune diseases (Zhang *et al.*, 2013). After stimulation Treg cells use contact-dependent mechanisms and secrete inhibitory cytokines such as IL10 and IL35 to contain effector T cell responses. CTLA4 on the surface of Treg cells competes with CD28 on effector T cells for the binding of CD80 and CD86 on the surface of antigen-presenting cells and thereby limits effector T cell activation. In a comparable manner CD226 and TIGIT compete for the binding of CD155 on the surface of antigen-presenting cells, but the importance of these interactions for the co-stimulation and co-inhibition of Treg and effector T cells remains to be elucidated.

Reduced suppressive function of peripheral Treg cells has been recorded in autoimmune disorders (Viglietta *et al.*, 2004; Buckner, 2010), although the exact mechanism leading to reduced suppression is unknown. A number of genes have been linked with altered Treg function in patients with multiple sclerosis, among them genes encoding the IL-2 receptor alpha chain (*IL2RA*) (Dendrou *et al.*, 2009) and CD58 (De Jager *et al.*, 2009). Two single nucleotide polymorphisms located in the CD226 gene, rs727088 and rs763361, which are in strong linkage disequilibrium, have been identified to be associated with several autoimmune diseases and to influence *CD226* mRNA levels in healthy individuals (Löfgren *et al.*, 2010). The allelic combination of $rs727088^G$ and $rs763361^T$ represents the risk haplotype ('R'), whereas the haplotype $rs727088^A$ and $rs763361^C$ is considered protective for autoimmune diseases ('P') (Qiu *et al.*, 2013). However, the mechanism by which these genetic variants of CD226 predispose to autoimmunity and whether they might impact Treg function remains enigmatic.

Materials and methods

Peripheral blood donors

The study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg) and written consent was obtained from all healthy individuals and multiple sclerosis patients. Multiple sclerosis was diagnosed according to the 2010 revised McDonald criteria for diagnosis (Polman *et al.*, 2011). Patients designated as 'untreated' had not received disease-modifying treatment during at least 1 year before the time point of sample acquisition. Glatiramer acetate- and interferon-β-treated patients had been under treatment for at least 4 or 6 months, respectively. Detailed clinical characteristics of the cohorts of patients and healthy individuals used for CD226 expression analysis on peripheral blood cells are summarized in Supplementary Tables 1–4.

Single nucleotide polymorphism genotyping

We genotyped rs763361 and rs727088 by using TaqMan® SNP Genotyping Assay (Applied Biosystems) according to manufacturer's protocols. We scored the genotyping data independently by two investigators to minimize reporting error. Carriers of $rs763361^T$ and $rs727088^G$ haplotype were considered at risk ('R'), whereas $rs763361^C$ and $rs727088^A$ haplotype was termed protective haplotype ('P'). Due to close linkage disequilibrium we were not able to investigate both genetic loci separately. Individuals with alternative genotype combinations were excluded from this study.

Flow cytometry

We stained surface antigens of human peripheral blood cells for 30 min at 4°C, followed by lysis of erythrocytes and fixation of lymphocytes using BD FACS Lysing Solution (BD Biosciences) for 10 min at room temperature. We used the following fluorochrome-conjugated anti-human monoclonal antibodies in this study: CD3 (UCHT1, BD Biosciences); CD4 (RPA-T4, eBioscience); CD8a (DK25, Dako); CD25 (BC96, BioLegend); CD45RA (HI100, Life Technologies and BioLegend); CD56 (B159, BD Biosciences); CD69 (FN50, BD Biosciences); CD127 (A019D5, BioLegend); CCR7 (3D12, BD

Biosciences); CD226 (DX11, BD Biosciences); IgG1 (MOPC-21, BD Biosciences); FOXP3 (206D, BioLegend). For staining of mouse immune cells the following antibodies were used: CD3 (145-2C11, BioLegend); CD3 (500A2, BD Biosciences); CD4 (GK1.5 and RM4-5, eBioscience); CD8a (53-6.7, BioLegend); CD11b (M1/70, BioLegend); CD11c (N418, eBioscience); CD25 (PC61.5, eBioscience); CD39 (24DMS1, eBioscience); CD44 (IM7, BioLegend); CD45R (RA3-6B2, eBioscience); CD62L (MEL-14, eBioscience); CD69 (H1.2F3, eBioscience); CD73 (eBioTY/11.8, eBioscience); CTLA-4 (UC10-4B9, eBioscience); CD226 (480.1; BioLegend); FOXP3 (FJK-16s, eBioscience); GARP (YGIC86, eBioscience); GITR (DTA-1, eBioscience); Helios (22F6, BioLegend); LAP (TW7-16B4, BioLegend); Ly-6G (1A8, BD Biosciences); Neuropilin-1 (3E12, BioLegend); NK1.1 (PK136, eBioscience); TCR Vα3.2 (RR3-16, BD Biosciences); TIGIT (GIGD7, eBioscience). We stained intracellular FOXP3 with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. For peripheral blood mononuclear cell staining, we excluded dead cells from analysis by staining with LIVE/DEAD® Fixable Aqua or Near-IR Dead Cell Stain Kit (Life Technologies) following the manufacturer's protocols. We obtained data using a BD LSR II flow cytometer (BD Biosciences) and analysed them by using FlowJo (Tree Star).

Analysis of *CD226* mRNA expression

We purified RNA from total peripheral blood mononuclear cells by using RNeasy® Mini Kit (Qiagen) including homogenization with QIAshredder columns (Qiagen) according to the manufacturer's protocol. Two hundred nanograms of total RNA were reverse transcribed into cDNA using oligo(dT) primers and the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). We determined *CD226* mRNA levels by quantitative real-time PCR using TaqMan® Gene Expression Assay Hs00170832_m1 (LifeTechnologies), normalizing to TATA box binding protein (*TBP*, Hs99999910_m1). All samples were run in triplicates and relative expression was calculated as $2^{-\Delta Ct}$.

Stimulation of human cells

We obtained peripheral blood mononuclear cells by Ficoll gradient centrifugation from EDTA blood drawn from voluntary healthy blood donors and patients with multiple sclerosis in independent experiments. We stimulated total peripheral blood mononuclear cells with 1 µg/ml antibody directed against CD3 (OKT3, BioXCell) and analysed CD226 and CD69 expression either on $CD4^+FOXP3^-$ or $CD4^+CD25^{high}FOXP3^+$ cells by flow cytometry at different time points. Naïve and memory T cell populations were distinguished based on their CD45RA expression ($CD45RA^+$: naïve; $CD45RA^-$: memory). For every day of analysis individuals of all three different genotypes were used. Results of individual experiments were afterwards pooled by genotype for statistical analysis. Due to the fact that stimulation experiments with healthy individuals and patients with multiple sclerosis were performed independently several months apart, different CD226 antibody batches and different flow cytometer settings had to be used, so that median fluorescent intensities (MFIs) of CD226 cannot directly be compared between patients with multiple sclerosis and healthy individuals in these experiments.

Human regulatory T cell suppression assay

For Treg suppression assays using human immune cells, we sorted $CD4^+CD25^{high}CD127^-$ regulatory T cells by fluorescence-activated cell sorting (FACS), using a BD FACSAria III (BD Biosciences) from peripheral blood mononuclear cells of healthy individuals or untreated patients with multiple sclerosis either homozygous for the protective or risk *CD226* haplotype. Subsequently, we mixed these cells with peripheral blood mononuclear cells of a separate but always the same healthy individual, which had been fluorescently labelled with Cell Proliferation Dye eFluor® 670 (eBioscience) according to manufacturer's instructions. We determined the ratio of peripheral blood mononuclear and Treg cells by flow cytometry and adjusted it to 1:1. Treg concentrations were subsequently serially diluted. We stimulated mixed cultures with plate-bound anti-CD3-antibody (OKT3, BioXCell) at a concentration of 0.5 µg/ml for 69–72 h. We quantified proliferation by flow cytometry and calculated the percentage of initially proliferating cells using FlowJo software (TreeStar).

Mice

$Cd226^{-/-}$ mice (Gilfillan *et al.*, 2008), $Cd155^{-/-}$ (also known as $Pvr^{-/-}$) mice (Maier *et al.*, 2007) and 2D2 transgenic mice (Bettelli *et al.*, 2003) were previously described. We used female and male $Cd226^{-/-}$, $Cd155^{-/-}$ and respective littermate control mice on a C57BL/6 background with all experiments being approved by the local ethics committee (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz in Hamburg; G09/121 and G12/50).

Experimental autoimmune encephalomyelitis induction

We induced EAE in sex- and age-matched 6–12-week-old mice as previously described (Schattling *et al.*, 2012). In brief, we injected subcutaneously 100 µg myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}; Schafer-N) emulsified in complete Freund's adjuvant (BD Difco) containing heat-inactivated *Mycobacterium tuberculosis* (BD Difco) at 2 mg/ml into two sites of the hind flanks, followed by intravenous injection of 100 ng pertussis toxin (*Bordetella pertussis*; Merck Biosciences) in phosphate-buffered saline (PBS), which was repeated 48 h later. We scored mice daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and fore limb paresis; 5, pre-morbid or dead. We sacrificed animals at a score $\geqslant 4$.

Regulatory T cell transfer

Inguinal, axillary, brachial, cervical lymph nodes and spleens were harvested with sterile instruments and collected into ice cold PBS. Single cell suspension was prepared by homogenization through a 40 µm cell strainer. Treg cells were isolated from single cell suspensions using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Purity of resulting $CD4^+CD25^-$ effector T cell and $CD4^+CD25^+$ Treg populations was

routinely above 90%. To increase Treg numbers for transfer, Treg *in vitro* expansion was performed as previously described (Kruisbeek *et al.*, 2004). Briefly, Treg cells were cultured in complete T cell medium containing 100 U/ml IL2 at a cell density of 1×10^6 cells/ml in anti-CD3-coated 24 well plates. Treg cells were split on Day 3 and harvested on Day 7 for consecutive transfer of $2–3 \times 10^6$ cells into wild-type recipients. The day after transfer, EAE was induced in recipient animals as described above.

Stimulation assay with mouse cells

We isolated splenocytes of 2D2 transgenic mice and stimulated them with 10 µg/ml MOG_{35-55} (Schafer-N) for 24–72 h. We analysed CD226 expression by flow cytometry after gating on $CD4^+$ TCR $V\alpha3.2^+$ T cells.

Assays for mouse regulatory T cell function

We conducted Treg suppression assays for mouse cells as described elsewhere (Kruisbeek *et al.*, 2004). In brief, we isolated $CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ effector T cells using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. For feeder cells, we used T cell-depleted splenocytes. Therefore, we incubated splenocytes with anti-CD90.2 (clone 30-H12, BioLegend) antibody and Low-Tox-M rabbit complement (Cedarlane) for 30 min at 37°C and subsequently irradiated the resulting cells with 40 Gy. We added 50 000 effector T cell cells to 50 000 feeder cells and decreasing numbers of Treg cells starting from 50 000 Treg cells (1:1) to 3125 Treg cells (16:1). We stimulated the cells subsequently with 0.25 µg/ml anti-CD3 (2C11, BioLegend) for 72 h, before they were pulsed by addition of 1 µCi ^{3}H-thymidine per well for additional 16 h. We quantified incorporated ^{3}H-thymidine using a beta-counter (Perkin-Elmer). For quantification of Treg proliferation, we isolated $CD4^+CD25^+$ regulatory T cells, stimulated them for 72 h with 0.25 µg/ml anti-CD3 antibody (2C11; BioLegend) in presence of either 0.5 µg/ml anti-CD28 antibody (37.51; BioLegend) or 50 U/ml IL2 (eBioscience) and quantified proliferation by ^{3}H-thymidine incorporation as described above. We repeated all assays at least three times.

Statistical analysis

We performed all statistical tests using GraphPad Prism and SPSS. All values are expressed as means ± SEM. Where indicated, we analysed for significance by using Mann-Whitney U-test for two groups, by ANOVA with appropriate *post hoc* analysis for multiple groups or by Kruskal-Wallis test for non-parametric analysis for multiple groups. Regression analysis was used to test for correlations between *CD226* haplotype and CD226 protein expression, or between *Cd226* genotype of mice and frequencies of Treg cells among $CD4^+$ splenocytes, assuming a linear genotype–phenotype correlation. Survival of EAE mice was analysed by Mantel-Cox test. We considered $^*P < 0.05$ as significant, $^{**}P < 0.01$ and $^{***}P < 0.001$ as highly significant.

Results

$CD4^+$ T cells of healthy *CD226* risk haplotype carriers show reduced CD226 expression

To assess phenotypical consequences of the variants in the *CD226* gene in the context of an autoimmune disease, we investigated CD226 expression on peripheral T cells of healthy individuals and patients with multiple sclerosis. In memory $CD4^+$ T cells, which in contrast to naïve $CD4^+$ T cells exhibit high expression levels of CD226 (Fig. 1A), we found that the presence of the risk haplotype correlated with a reduced expression of the CD226 protein on the cell surface in a gene dosage effect in healthy individuals ($P = 0.004$; Fig. 1B and Supplementary Table 1). By contrast, we found that this genotype–phenotype correlation was absent in patients with multiple sclerosis, where surface expression of CD226 on memory $CD4^+$ T cells and mRNA expression of CD226 in total peripheral blood mononuclear cells did not significantly differ between protective and risk haplotype carriers (Fig. 1B, Supplementary Table 1 and Supplementary Fig. 1A). Of note, patients with relapsing-remitting multiple sclerosis showed overall a reduced CD226 expression in comparison to healthy individuals ($P = 0.012$) or secondary-progressive multiple sclerosis patients ($P = 0.024$; Supplementary Fig. 1B) with equal distribution of the respective genotypes among the different groups. In naïve $CD4^+$ T cells, we did not detect haplotype dependent differences in CD226 expression in both healthy individuals and patients with multiple sclerosis (Fig. 1C).

As CD226 expression is upregulated during T cell activation (Lozano *et al.*, 2012), reaching peak CD226 expression level after 48 h of polyclonal TCR stimulation (Fig. 1D and E), we next analysed whether activation-induced CD226 increase is haplotype-dependent. In naïve T cells of healthy individuals and patients with multiple sclerosis we did not detect significant differences in CD226 expression after stimulation (Fig. 1D). Notably, in healthy individuals $CD4^+$ memory T cells of protective haplotype carriers almost doubled their CD226 expression at peak expression ($P = 0.04$). By contrast, T cells of heterozygous and homozygous risk haplotype carriers failed to significantly increase CD226 expression after polyclonal TCR stimulation (Fig. 1E), resulting in reduced CD226 expression levels on T cells of healthy homozygous risk haplotype carriers after stimulation. In multiple sclerosis patients, memory $CD4^+$ T cells from both homozygous protective and homozygous risk haplotype carriers did not significantly upregulate CD226 in response to stimulation (Fig. 1E). Differences in upregulation of CD226 were irrespective of activation status in these assays, as the different genotypes showed identical induction of CD69 surface expression in both healthy individuals and multiple sclerosis patients (Supplementary Fig. 1C).

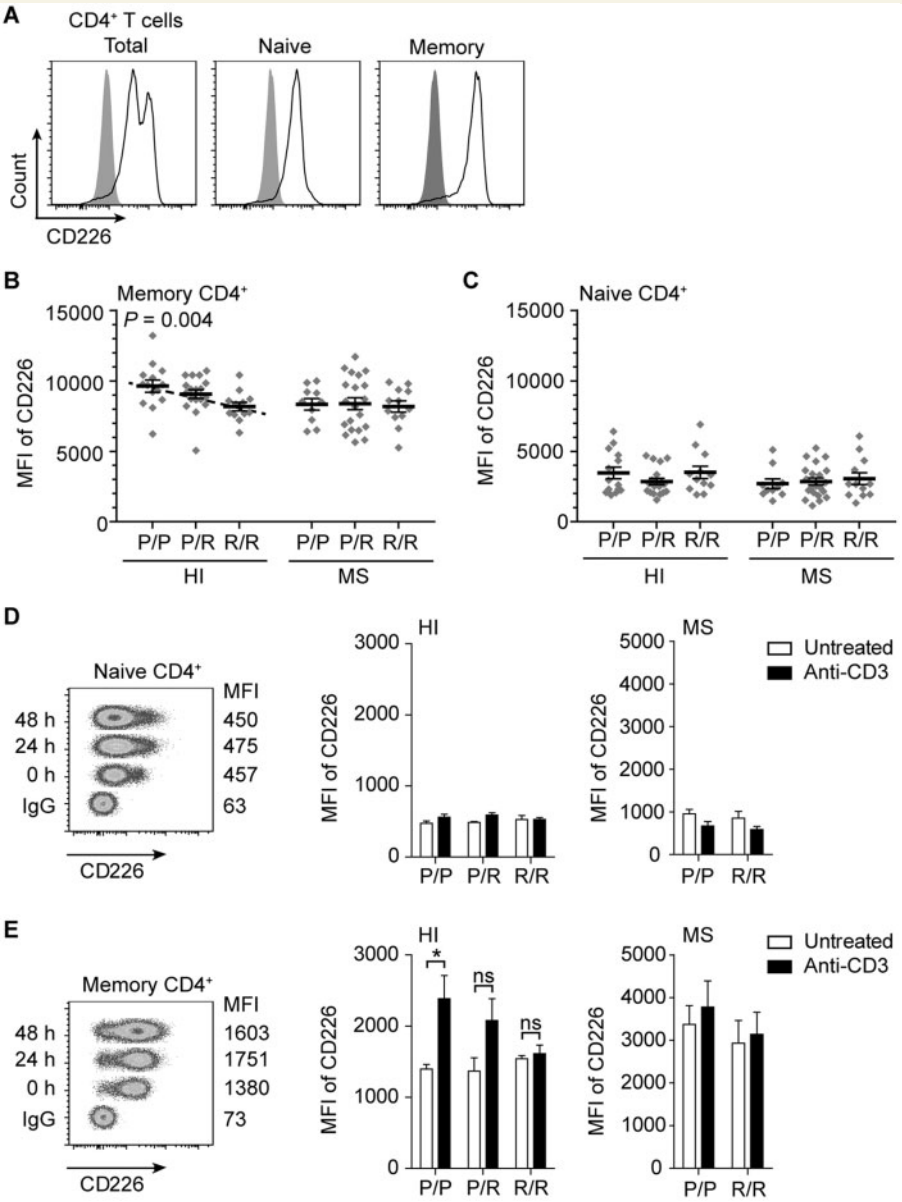

Figure 1 *CD226* risk haplotype is associated with reduced CD226 expression on memory and activated CD4$^+$ T cells in healthy individuals. (**A**) Representative staining of peripheral blood cells for CD226 on total CD4$^+$ T cells (CD56$^-$CD4$^+$; *left*) and on naïve (CD56$^-$CD4$^+$CD45RA$^+$; *middle*) and memory CD4$^+$ T cells (CD56$^-$CD4$^+$CD45RA$^-$; *right*). Open histogram: CD226 antibody staining; filled area: isotype antibody staining. (**B** and **C**) Median fluorescence intensity (MFI) of CD226 expression on memory (**B**) and naïve (**C**) CD4$^+$ T cells from healthy individuals (HI) and patients with multiple sclerosis (MS) of indicated genotype (P = protective; R = risk; P/P: n = 14; P/R: n = 18; R/R: n = 12 for healthy individual and P/P: n = 10; P/R: n = 20; R/R: n = 12 for multiple sclerosis). Error bars represent ± SEM. Interaction was analysed by linear regression analysis assuming linear genotype–phenotype correlation. (**D** and **E**) Peripheral blood mononuclear cells were stimulated with anti-CD3 antibody. CD226 staining of CD4$^+$FOXP3$^-$CD45RA$^+$ naïve T cells (**D**) and CD4$^+$FOXP3$^-$CD45RA$^-$ memory T cells (**E**) after indicated duration of stimulation of one representative, homozygous protective haplotype carrier is shown (*left*). IgG represents isotype control staining. MFI ± SEM of untreated peripheral blood mononuclear cells (0 h) and 48 h after stimulation of n = 3 healthy individuals (*middle*) and n = 5 multiple sclerosis patients (*right*) of indicated *CD226* genotype are depicted. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* analysis; *$P < 0.05$.

CD226 is expressed on memory regulatory T cells and reduced in healthy risk haplotype carriers

Given that CD226 has been described to act as activating co-signalling molecule (Gilfillan *et al.*, 2008), we next rationalized that a reduction in CD226 surface expression could predispose to autoimmunity, if this phenotypical alteration affected the inhibitory function of $CD4^+$ Treg cells. Therefore, we next conducted CD226 surface staining including markers to distinguish regulatory T cells. Here we found that memory Treg cells stained positive for CD226 (Fig. 2A), albeit with considerably lower intensity than $FOXP3^-$ T cells (Fig. 2B and Supplementary Fig. 2A). CD226 expression was virtually absent on naïve Treg cells, suggesting a possible unrecognized function of CD226 particularly in memory Treg cells. Thus, we analysed haplotype-dependent CD226 expression on memory Treg cells of healthy individuals and patients with multiple sclerosis. In healthy individuals we found a significantly reduced surface expression in homozygous but also in heterozygous risk haplotype carriers in comparison to homozygous protective haplotype carriers ($P = 0.02$ and $P = 0.03$, respectively; Fig. 2B and Supplementary Table 2). These findings are consistent with the genotype–phenotype correlation observed for total memory $CD4^+$ T cells of healthy individuals (Fig. 1B). Again, we recorded an abrogated haplotype-dependent reduction of CD226 surface expression on memory Treg cells in patients with multiple sclerosis (Fig. 2B and Supplementary Table 2) as cells from all three genotype carriers exhibited median fluorescent intensities that were similar to those observed in cells from healthy risk haplotype carriers. There were no differences in CD226 expression on naïve Treg cells (Supplementary Fig. 2B) and Treg frequencies in the same groups (Supplementary Fig. 2C).

To get an insight whether the co-stimulatory activity of CD226 also influences Treg cells and if its reduction might contribute to the increased risk of autoimmunity, we next investigated stimulus-dependent CD226 induction in Treg cells of healthy individuals and patients with multiple sclerosis. After polyclonal stimulation with anti-CD3 antibody memory Treg cells of both groups increased their CD226 expression substantially, reaching peak CD226 expression levels after 24 h, which decreased thereafter (Fig. 2C and D). Notably, in healthy individuals risk haplotype carriers showed a reduced stimulation-induced peak expression of CD226 in memory Treg cells in comparison to Treg cells from homozygous protective haplotype carriers ($P < 0.001$; Fig. 2D). By contrast, in patients with multiple sclerosis stimulation-induced peak expression of CD226 in memory Treg cells of homozygous risk haplotype carriers was not significantly different in comparison to homozygous protective haplotype carriers (Fig. 2D), again indicating that haplotype specific differences in CD226 induction seen in healthy individuals are absent or less pronounced in patients with multiple sclerosis. The activation status as measured by CD69 upregulation was not different in the different groups (Supplementary Fig. 3).

Regulatory T cells of healthy risk haplotype carriers show impaired suppressive capacity

We next investigated, whether reduced CD226 expression on Treg cells in healthy risk haplotype carriers has functional consequences for their suppressive capacity. Indeed, total Treg cells of healthy homozygous *CD226* risk haplotype carriers were significantly impaired in their capacity to inhibit the proliferation of effector T cell cells in comparison to Treg cells of homozygous protective haplotype carriers ($P = 0.008$; Fig. 3A). Of note, this haplotype-dependent effect was absent in patients with multiple sclerosis with comparable suppressive capacity of Treg cells in homozygous protective and risk haplotype carriers (Fig. 3B).

As glatiramer acetate and interferon-β have been shown to alleviate impaired Treg function in patients with multiple sclerosis (de Andrés *et al.*, 2007; Korporal *et al.*, 2008; Haas *et al.*, 2009; Namdar *et al.*, 2010), we next analysed haplotype-dependent CD226 expression on Treg cells of patients with multiple sclerosis receiving these treatments. In both, glatiramer acetate- and interferon-β-treated patients (Supplementary Tables 3 and 4) we found a tendency towards higher CD226 expression levels in memory $CD4^+$ Treg cells of homozygous protective haplotype carriers in comparison to heterozygous and homozygous risk haplotype carriers (Fig. 3C and D). In interferon-β-treated patients with multiple sclerosis the difference between homozygous protective and heterozygous haplotype carriers was statistically significant ($P = 0.007$, Fig. 3D). Therefore, therapies capable of improving Treg function of patients with multiple sclerosis might be able to restore the haplotype–phenotype correlation of CD226 that is detected in healthy individuals.

Cd226-deficient mice show elevated frequencies of regulatory T cells with reduced suppressive activity

Considering the heterogeneity of human Treg responses, likely due to many different functional variants in the human genome (Lappalainen *et al.*, 2013), of which many impact Treg function (De Jager *et al.*, 2009; Dendrou *et al.*, 2009; Kofler *et al.*, 2011; Sawcer *et al.*, 2011) and the limitation of functional studies with human material, we next reduced complexity and mimicked phenotypically the diminished CD226 expression by using *Cd226*-deficient and *Cd226*-haplodeficient mice. Mouse and human CD226 are 53% homologous on amino acid level with conservation of the main signalling motives as well as binding to the same ligands (CD155 and CD112), and its function is largely conserved among species (Tahara-Hanaoka *et al.*, 2005). Consistently, mouse

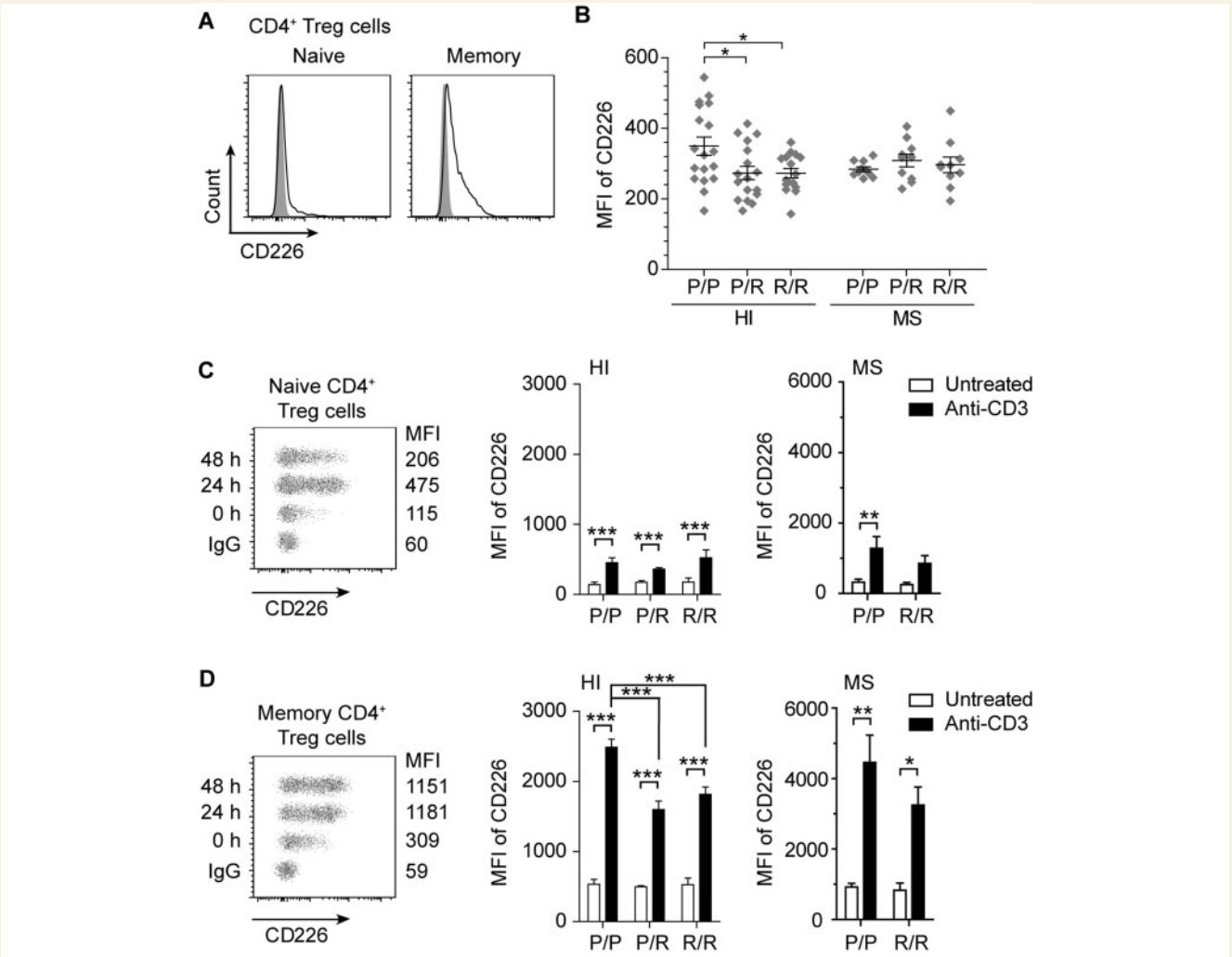

Figure 2 Regulatory T cells of healthy *CD226* risk haplotype carriers show reduced CD226 expression. (**A**) Representative overlay of CD226 staining (open histogram) on $CD4^+FOXP3^+CD45RA^+$ naïve Treg cells (*left*), $CD4^+FOXP3^+CD45RA^-$ memory Treg cells (*right*) and isotype antibody staining (grey histogram) of one healthy individual (HI). (**B**) Median fluorescence intensity (MFI) ± SEM of CD226 expression on memory Treg cells from healthy individuals and patients with multiple sclerosis of indicated genotype (P: protective; R: risk; $n = 17–18$ per genotype for healthy individuals and $n = 10$ per genotype for patients with multiple sclerosis). Statistical analysis was conducted by two-way ANOVA followed by Tukey's *post hoc* analysis; $^*P < 0.05$. (**C** and **D**) Freshly isolated peripheral blood mononuclear cells of healthy individuals or patients with multiple sclerosis were stimulated with anti-CD3 antibodies and $CD4^+FOXP3^+CD45RA^+$ naïve (**C**) and $CD45RA^-$ memory (**D**) Treg cells were analysed for CD226 expression after indicated time points. CD226 staining of one representative, homozygous risk haplotype carrier is shown (*left*). IgG represents isotype control staining. MFI ± SEM of untreated peripheral blood mononuclear cells (0 h) and 24 h after stimulation of $n = 3$ healthy individuals (*middle*) or $n = 5$ patients with multiple sclerosis (*right*) of indicated *CD226* genotype are depicted. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* analysis; $^{***}P < 0.001$; $^{**}P < 0.01$; $^*P < 0.05$.

$CD4^+$ T cell subsets showed CD226 expression patterns that were similar to human T cells, with high expression on memory $CD4^+$ T cells and activated T cells (Supplementary Fig. 4). Moreover, mouse wild-type effector T and Treg cells likewise upregulated CD226 after stimulation and corresponding cells from *Cd226*-haplodeficient mice remained low in CD226 expression after stimulation (Fig. 4A and B), similar to CD226 induction in human Treg cells from risk haplotype carriers (Fig. 2D).

Additionally, we found a *Cd226* gene dosage-dependent increase in the frequency of $FOXP3^+$ Treg cells among $CD4^+$ T cells isolated from spleens ($P = 3 \times 10^{-4}$; Fig. 4C). As we did not detect any differences in the subset composition of the Treg population with equal relative frequencies of thymus-derived Treg (tTreg) and peripherally induced Treg (pTreg) cells (Weiss *et al.*, 2012; Yadav *et al.*, 2012) among total $FOXP3^+$ Treg cells and no differences for other immune cell subsets (Supplementary Fig. 5), this observation provides additional evidence for a role of CD226 specifically in Treg cells.

As reduced CD226 expression in human Treg cells was associated with impaired suppressive capacity, we next

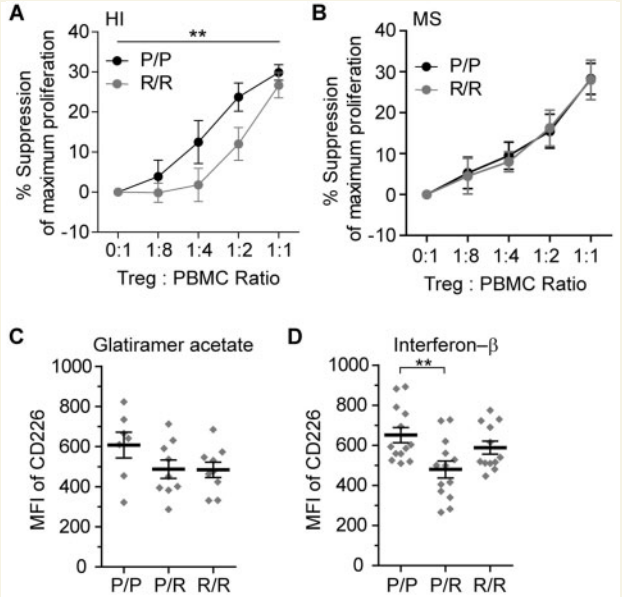

Figure 3 Treg cells of healthy CD226 risk haplotype carriers show reduced suppressive capacity. (**A** and **B**) Sorted Treg cells of healthy (**A**) or multiple sclerosis (**B**) homozygous protective (P/P) or risk (R/R) haplotype carriers ($n = 5$ per genotype) were mixed with allogeneic peripheral blood mononuclear cells (PBMCs) of one healthy individual at indicated ratios and stimulated with anti-CD3 antibody. Proliferation of $CD4^+$ T cells was quantified by eFluor670-dilution and normalized to proliferation of $CD4^+$ T cells without Treg cell addition. Shown are per cent suppression of maximum proliferation ± SEM. Statistical analysis was conducted by two-way ANOVA; $^{**}P < 0.01$. (**C** and **D**) Median fluorescence intensity (MFI) ± SEM of CD226 expression on memory Treg cells from patients with multiple sclerosis of indicated genotype under ongoing treatment with glatiramer acetate ($n = 7$–9 per genotype; **C**) or interferon-β ($n = 12$–13 per genotype; **D**). Statistical analysis was conducted by two-way ANOVA followed by Tukey's *post hoc* analysis; $^{**}P < 0.01$.

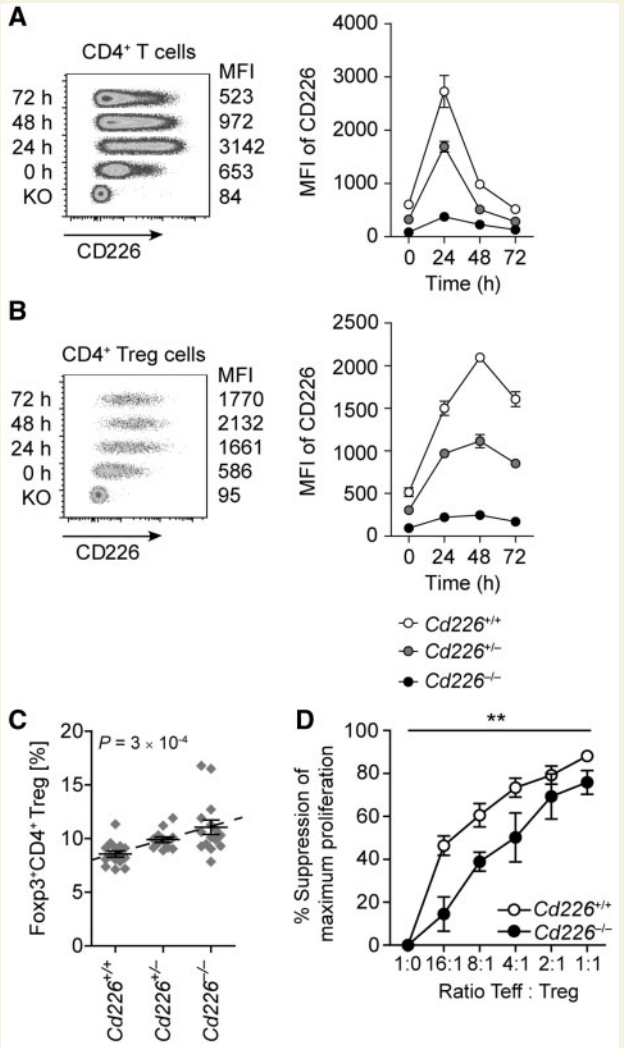

Figure 4 *Cd226*-deficiency leads to increased frequencies of Treg cells with reduced suppressive capacity. (**A** and **B**) We stimulated mouse splenocytes of indicated *Cd226* genotypes with anti-CD3 antibody and analysed CD226 expression on $FOXP3^-CD4^+$ (**A**) and $FOXP3^+CD4^+$ Treg cells (**B**) at the indicated time points. Shown are median fluorescence intensities (MFI) ± SEM of $n = 5$ mice per group. (**C**) Treg frequency among total $CD4^+$ T cells from splenocytes of mice with the indicated *Cd226* genotype ($n = 9$–15 mice per group; mean ± SEM). Interaction was analysed by linear regression analysis assuming linear phenotype–genotype correlation. (**D**) Purified $CD4^+CD25^{high}$ Treg cells from mice with indicated genotype were mixed with wild-type $CD4^+CD25^-$ effector T cells (Teff) at defined ratios and stimulated with anti-CD3 antibody. Proliferation was quantified by ^{3}H-thymidine incorporation ($n = 4$ per genotype). Statistical analysis was performed by two-way ANOVA; $^*P < 0.01$. KO = knock out.

investigated the inhibitory functionality of *Cd226*-deficient Treg cells. Indeed, in comparison to wild-type Treg cells, *Cd226*-deficient Treg cells were significantly less capable of suppressing the proliferation of wild-type effector T cells ($P = 0.04$; Fig. 4D). Of note, *Cd226*-deficient and *Cd226*-proficient Treg cells showed the same proliferation and surface expression of proteins important for Treg function (Supplementary Fig. 6), suggesting that expression level of CD226 itself regulates Treg function and not compensatory mechanisms.

CD226 deficiency leads to exacerbated experimental autoimmune encephalomyelitis

Having established an important function of CD226 for the regulation of the suppressive capacity of Treg cells *in vitro*, we next analysed whether a defect in this pathway might predispose to exaggerated autoimmune reactions *in vivo*. Consistently, *Cd226*-deficiency resulted in an exacerbated EAE disease course. We sacrificed mice that suffered from a prolonged period of paresis and paralysis of fore limbs (equivalent to a clinical score of 3.75 or 4). These mice were only included in the analysis until their day of

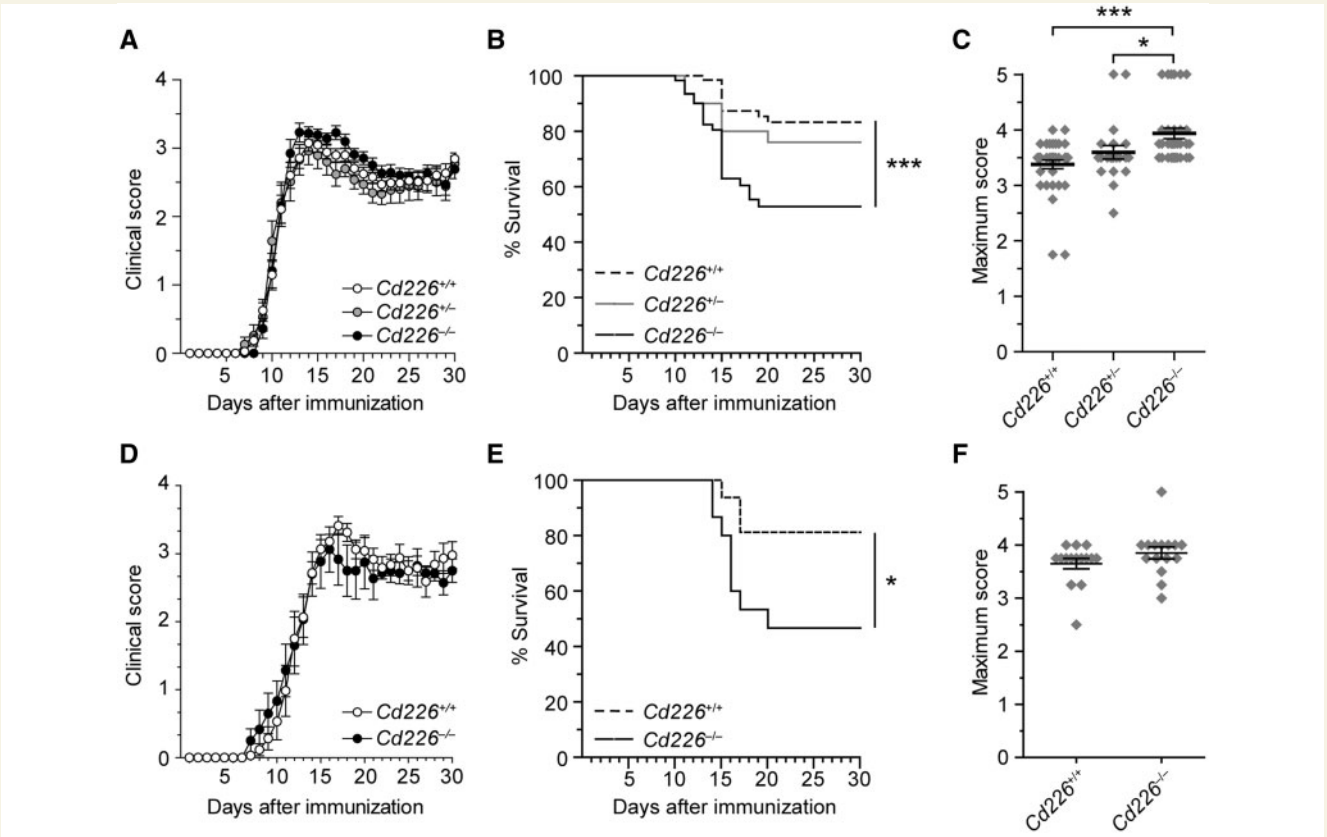

Figure 5 CD226 deficiency leads to enhanced autoimmunity. (**A**–**C**) We induced EAE in mice of indicated genotype (n = 21–37 per group). (**A**) Clinical scores are depicted as mean ± SEM. (**B**) Survival curves were analysed by Mantel-Cox test. (**C**) Maximum clinical scores are depicted as mean ± SEM with statistical analysis by non-parametrical Kruskal-Wallis test and subsequent Mann-Whitney U-test. (**D**–**F**) We induced EAE in wild-type C57BL/6 mice (n = 15–16 per group) after transfer of 2–3 × 10^6 Treg cells from either *Cd226*$^{+/+}$ or *Cd226*$^{-/-}$ mice. (**D**) Clinical scores are depicted as mean ± SEM. (**E**) Survival curves were analysed by Mantel-Cox-test. (**F**) Maximum clinical scores are depicted as mean ± SEM. Statistical analysis was performed by Mann-Whitney U-test. *$P < 0.05$, ***$P < 0.001$.

death, whereafter they were excluded from analysis to avoid bias by sacrificed mice. Due to this ceiling effect, the course of EAE was only slightly exacerbated in *Cd226*-deficient mice (Fig. 5A), but characterized by a pronounced reduction of the survival rate ($P = 0.0008$; Fig. 5B) and a significant increase in mean maximum score of *Cd226*$^{-/-}$ mice compared to respective littermate controls ($P < 0.001$; Fig. 5C). *Cd226*-haplodeficient mice developed an intermediate disease severity ($P = 0.004$) emphasizing the importance of the gene dose. To further investigate whether the functional deficiency of *Cd226*$^{-/-}$ Treg cells we observed in the *in vitro* suppression assays also holds true in the *in vivo* EAE setting, we performed adoptive transfers of Treg cells from *Cd226*$^{-/-}$ and wild-type donor mice into wild-type recipients. Consecutively, we induced EAE in recipient animals and assessed the influence of the Treg donor genotype on the development of disease. Indeed, recipients of *Cd226*$^{-/-}$ Treg cells showed a reduced survival in comparison to recipients of wild-type Treg cells (Fig. 5D–F; $P = 0.0379$), thus further supporting the notion of a relevant functional deficiency of *Cd226*$^{-/-}$ Treg cells.

Discussion

We identified that healthy individuals carrying autoimmunity predisposing genetic variants in the *CD226* gene show a reduced CD226 expression on CD4$^+$ T cells, which particularly affects CD4$^+$FOXP3$^+$ Treg cells in their suppressive capacity. In patients with multiple sclerosis, CD226 expression and suppressive capacity of Treg cells did not differ between carriers of the different genetic variants, implicating that in an on-going autoimmune disease protective haplotype effects are abrogated. Genetic polymorphisms in the gene encoding *CD226* have been associated with a variety of autoimmune diseases (Qiu *et al.*, 2013). Most of them, such as rheumatoid arthritis, type 1 diabetes and multiple sclerosis, are caused by dysregulated T cell responses pointing towards a shared affected pathway in T cell regulation involving CD226.

Although previous studies have connected an increased CD226 expression with potentially proinflammatory Th1 (Dardalhon *et al.*, 2005) and Th17 cells (Lozano *et al.*, 2013) and showed that antibody-mediated targeting of

CD226 ameliorated the course of EAE (Dardalhon *et al.*, 2005), our data support a model in which CD226 is particularly important for the function of Treg cells. This also best explains why a reduced surface expression of a co-stimulatory molecule is associated with autoimmunity. Treatment of animals with CD226-antibody potentially blocks cells with high CD226 expression. As we and others show that CD226 is upregulated during activation of encephalitogenic $CD4^+$ T cells (Lozano *et al.*, 2012), antibody treatment most likely affects or even depletes effector T cells and thereby reduces EAE severity. In our model using CD226-deficient mice we can more precisely mimic the genetically encoded reduced CD226 expression observed in healthy risk haplotype carriers and investigate resulting effects. We are able to delineate the importance of CD226 expression level for the full function and suppressive capacity of Treg cells, which is compromised in healthy humans carrying the CD226 risk haplotype as well as in mice with reduced CD226 expression.

By contrast, in acute graft-versus-host disease CD226 serves a critical role in $CD8^+$ donor cells, whereas CD226 is dispensable for CD4 T cell function (Nabekura *et al.*, 2010). Contradictory to these results, Koyama and colleagues (2013) proposed that CD226 inhibits the suppressive capacity of Treg cells in a setting of graft-versus-host disease. Whether the tightly regulated balance between co-stimulation through CD226 and co-inhibition through TIGIT is differently affected in Treg cells during graft-versus-host disease and autoimmunity needs subsequent studies.

Notably, except for the alteration in mouse Treg frequencies among splenocytes and the reduced inhibition of effector T cell responses, we did not detect other alterations in the expression of surface markers previously associated with Treg suppressive function. The elevation in Treg frequency points towards compensatory mechanisms limiting the impaired suppressive capacity of Treg cells to prevent dysregulated autoimmune reactions to host tissues. However, these do not fully compensate the phenotype as *CD226*-deficient EAE mice still show a more severe disease course. Furthermore, our results indicate that CD226 itself is important for Treg function and that CD226 reduction does not evoke compensatory regulation of other molecules, which could explain the observed effects caused by reduced CD226 expression. Importantly, the reduced suppressive capacity of *Cd226*-deficient Treg also translated into EAE, resulting in reduced survival of animals that received *Cd226*-deficient in comparison to *Cd226*-proficient Treg cells. Thus, loss of CD226 expression appears to most prominently affect the Treg population rendering them incapable of properly controlling autoimmune effector responses during EAE *in vivo*.

Moreover, analogous observations for a diverse role in effector T cells and Treg cells were made for CD28, an important co-stimulatory factor, which was recently shown to be an obligate factor for full inhibitory capacity of Treg cells (Zhang *et al.*, 2013), whereas its ligands, CD80 and CD86, serve redundant functions, with their contribution only being detectable in mice lacking both ligands simultaneously (Zhang *et al.*, 2013). Of note, CD155, one of two described ligands of CD226 (Bottino *et al.*, 2003), has a similarly redundant function in this regulatory mechanism, as $Cd155^{-/-}$ EAE mice failed to show a similar phenotype as $Cd226^{-/-}$ EAE mice (Supplementary Fig. 7).

It has been shown that CD226 interacts upon activation with the adhesion molecule LFA-1 and is located in the peripheral supramolecular adhesion complex (p-SMAC) surrounding the central SMAC containing the TCR, CD3 and CD28 (Shirakawa *et al.*, 2006). Thereby it serves an important function to stabilize the interaction between cytotoxic T cells and target cells to license the killing of infected cells (Ramsbottom *et al.*, 2014). Similar to effector T cells, Treg cells depend on tight interaction with antigen-presenting cells to receive co-stimulatory signals (Herman *et al.*, 2012) and to influence antigen-presenting cells and effector T cells in an inhibitory, contact-dependent manner. Therefore, molecularly, CD226 deficiency in Treg cells might impair the interaction of Treg cells with CD155/CD112-expressing antigen-presenting- and T cells resulting in a reduced suppressive capacity.

Other genetic variants associated with autoimmune diseases, such as in the genes encoding *IL2RA* (Hafler *et al.*, 2007; Dendrou *et al.*, 2009) and *CD58* (De Jager *et al.*, 2009), equally result in an altered function of Treg cells, underlining that several pathways feed into Treg dysfunction and autoimmunity. Moreover, considering that patients with an ongoing autoimmune disease such as multiple sclerosis, acquired a CD226 expression level comparable to risk haplotype carriers irrespective of their haplotype and showed no haplotype-dependent differences in Treg suppressive capacity, it is intriguing to speculate that weakened co-signalling via CD226 might partially explain functional impairment of Treg cells in patients with multiple sclerosis (Viglietta *et al.*, 2004). Consistently, the haplotype–phenotype effect on CD226 expression was partially restored in interferon-β-treated patients with multiple sclerosis, where homozygous protective haplotype carriers again showed increased CD226 expression. Interferon-β treatment is known to positively influence Treg function (de Andrés *et al.*, 2007; Korporal *et al.*, 2008; Haas *et al.*, 2009; Namdar *et al.*, 2010). Currently, it is unknown how other non-genetically acquired mechanisms might contribute to a reduction in CD226 expression and whether a low CD226 expression might serve as prognostic marker for autoimmune diseases.

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Supplementary material

Supplementary material is available at *Brain* online.

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