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Developmental endothelial locus-1 (Del-1) is a homeostatic factor in the central nervous system limiting neuroinflammation and demyelination

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Abstract

Inflammation in the central nervous system (CNS) and disruption of its immune privilege are major contributors to the pathogenesis of multiple sclerosis (MS) and of its rodent counterpart,

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experimental autoimmune encephalomyelitis (EAE). We have previously identified developmental endothelial locus-1 (Del-1) as an endogenous anti-inflammatory factor, which inhibits integrin-dependent leukocyte adhesion. Here we show that Del-1 contributes to the immune privilege status of the CNS. Intriguingly, Del-1 expression decreased in chronic active MS lesions and in the inflamed CNS in the course of EAE. Del-1-deficiency was associated with increased EAE severity, accompanied by increased demyelination and axonal loss. As compared to control mice, Del-1$^{-/-}$ mice displayed enhanced disruption of the blood brain barrier and increased infiltration of neutrophil granulocytes in the spinal cord in the course of EAE, accompanied by elevated levels of inflammatory cytokines, including IL-17. The augmented levels of IL-17 in Del-1-deficiency derived predominantly from infiltrated CD8^{+} T cells. Increased EAE severity and neutrophil infiltration due to Del-1-deficiency was reversed in mice lacking both Del-1 and IL-17-receptor, indicating a crucial role for the IL-17/neutrophil inflammatory axis in EAE pathogenesis in Del-1$^{-/-}$ mice. Strikingly, systemic administration of Del-1-Fc ameliorated clinical relapse in relapsing-remitting EAE. Therefore, Del-1 is an endogenous homeostatic factor in the CNS protecting from neuroinflammation and demyelination. Our findings provide mechanistic underpinnings for the previous implication of Del-1 as a candidate MS susceptibility gene and suggest that Del-1-centered therapeutic approaches may be beneficial in neuroinflammatory and demyelinating disorders.

Introduction

The hallmark of neuroinflammatory demyelinating diseases in the central nervous system (CNS), such as multiple sclerosis (MS), is exacerbated inflammatory cell accumulation. Under normal conditions, the intact blood-brain barrier (BBB) prevents inflammatory cells from extravasating into the CNS. The BBB is thereby a component of the immune-privilege status of the CNS. In the course of MS and of its animal model, experimental autoimmune encephalomyelitis (EAE), the disruption of the BBB and the infiltration of autoreactive T cells, e.g. of the Th1 and Th17 lineages, and their respective cardinal cytokines, IFN-γ and IL-17, trigger a strong inflammatory response including the recruitment of further immune cells, such as neutrophils, monocytes/macrophages, and the activation of resident microglia, thereby leading to myelin damage[1, 2].

Regulation of leukocyte-endothelial interactions and immune cell recruitment represent an important therapeutic modality in EAE and MS[3-5]. For example, natalizumab, an antibody targeting the interaction between the leukocyte integrin VLA-4 and its endothelial counter-receptor VCAM-1, is an efficient treatment for MS[3, 6, 7]. In addition, we and others have shown that leukocyte function-associated antigen-1 (LFA-1) is involved in immune cell infiltration in the course of EAE progression, and that blocking the interaction between LFA-1 and its endothelial counter-receptor ICAM-1 ameliorates the severity of EAE[4, 5, 8, 9]. Whereas the majority of studies so far have focused on the activation of autoreactive and inflammatory cells in EAE and MS disease development, very little is known about alterations in homeostatic factors of the CNS that may counter-act MS/EAE pathogenesis.

We previously identified the endothelial cell-secreted developmental endothelial locus-1 (Del-1) as a novel endogenous homeostatic anti-inflammatory factor that interferes with

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leukocyte integrin beta2-integrin-dependent inflammatory cell adhesion to the endothelium[10-14]. Moreover, we recently showed that IL-17 can downregulate endothelial Del-1, thereby promoting LFA-1-dependent neutrophil recruitment and inflammatory bone loss[15]. Consistently, decreased Del-1 expression in men and mice was associated with elevated IL-17-dependent inflammation and inflammatory bone loss[15].

Intriguingly, the highest expression of Del-1 has been observed in the CNS[12] and Del-1 is a candidate MS susceptibility gene[16]. We have thus hypothesized that Del-1 acts as an endogenous homeostatic CNS factor that contributes to the immune privilege status of the CNS. This hypothesis is strongly supported by our present findings that Del-1 expression is reduced in MS and EAE, whereas Del-1-deficiency is associated with enhanced BBB disruption, an elevated inflammatory response and exacerbated EAE disease severity featuring increased demyelination.

Materials and Methods

Antibodies and Reagents

Antibody to mouse CD31 (clone 2H8) was purchased from AbDSerotec (Kidlington, UK) and rabbit polyclonal Antibody to NeuN was from Millipore (Darmstadt, Germany). Antibodies to neurofilament 200 and MBP were from Abcam (Cambridge, UK). Monoclonal antibody to β-gal (clone GAL-13) was from Sigma (St. Louis, MO). PE-conjugated anti-mouse CD45 (clone 30-F11), APC-conjugated anti-mouse CD11b (clone M1/70) and PE-conjugated anti-mouse Ly6G (clone 1A8) were from BD Biosciences (Heidelberg, Germany). FITC-conjugated anti-mouse CD4 (clone GK1.5) and PerCP-conjugated anti-mouse CD8 (clone 53-6.7) were from Miltenyi Biotec (Bergisch Gladbach, Germany). APC-conjugated anti-mouse F4/80 (clone BM8), FITC-conjugated anti-mouse IL-17A (clone eBio17B7) and APC-conjugated anti-mouse IFN-γ (clone XMG1.2) and FITC-conjugated anti mouse CD45 were from eBioscience (Frankfurt, Germany). Rat anti-mouse CD8α (clone 53-6.7) was from Novusbio (Littleton, CO). Rabbit polyclonal antibody to IL-17 was from Abcam (Cambridge, UK). Goat anti-rat Alexa 568 and goat anti-rabbit Alexa 488 were from Life Technologies (Darmstadt, Germany). Del-1-Fc was constructed, expressed, and purified as previously described[17].

Mice and induction of EAE

Del-1$^{-/-}$ mice, IL-17RA$^{-/-}$ and Del-1$^{-/-}$IL-17RA$^{-/-}$ in the C57BL/6 background were previously described[15]. Animal experiments were approved by the Landesdirektion Sachsen, Germany. Nine to twelve week old female mice were subcutaneously injected with 200 μg of MOG_{35-55} peptide (American Peptide Company, Sunyvale, CA) in incomplete Freund's adjuvant (Sigma) containing 500 μg inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI), immediately followed by intraperitoneal injection of 400 ng pertussis toxin (Merck, Darmstadt, Germany), following previous protocols[4, 18]. Two days later, the same quantity of pertussis toxin was injected. In the course of EAE development, mice were scored daily according to the following scale: 0, no clinical sign; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, limp tail and unilateral hind

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limb paralysis; 4, limp tail and bilateral hind limb paralysis; 5, four leg paralysis; 6, moribund or dead.

Relapsing and remitting EAE was induced in SJL/J mice (Charles River) that were immunized with 100 μg proteolipid protein peptide 139-151 ($PLP_{139-151}$; Genemed Synthesis) emulsified in complete Freund's adjuvant with 6 mg/ml *Mycobacterium tuberculosis* (Difco). Pertussis toxin (200 ng) was intraperitoneally injected into the mice on the day of immunization and 2 days later. 68 μg Del-1-Fc per mouse was intraperitoneally administered daily for four consecutive days after remission of first clinical RR-EAE attack. An equimolar amount of recombinant human IgG Fc (control-Fc; R&D Systems) was administered into control animals. All animals were kept under standard conditions and had access to water and food *ad libitum*. The clinical scores were measured by a blinded investigator using the following scoring system: 0, no abnormality; 1, limp tail tip; 2, limp tail; 3, moderate hind limb weakness; 4, complete hind limb weakness; 5, mild paraparesis; 6, paraparesis; 7, heavy paraparesis or paraplegia; 8, tetraparesis; 9, quadriplegia or premoribund state; 10, death. Relapsing-remitting experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany.

Further detailed Methods (Cell culture, Immunohistochemistry, Isolation of CNS inflammatory cells and flow cytometry analysis, Intracellular cytokine staining, *In vivo* BBB permeability assay, Real-time RT-PCR, Statistical analysis) are included in the Supplementary Information.

Results

Del-1 expression in the inflamed CNS of MS patients and of mice subjected to EAE

We have previously shown that the highest expression of the endogenous anti-inflammatory factor, Del-1, is found in the brain[12]. Here, we set out to analyze the expression of Del-1 in the brain in detail. To this end, we first examined the expression pattern of Del-1 by performing β-galactosidase (β-gal) staining in the brain of wild-type (WT) and Del-$1^{-/-}$ mice. It should be noted that Del-$1^{-/-}$ mice contain a LacZ knock-in and the LacZ gene is controlled by the Del-1 promoter, thereby serving as a reporter for Del-1 expression[12]. Del-$1^{-/-}$ brains displayed abundant β-gal-staining, especially in the cerebellum and the hippocampus, whereas as expected no β-gal staining could be detected in WT brains (Figure 1a). Given the expression of Del-1 in endothelial cells in several other tissues[12, 15, 19, 20], endothelial cells in the brain were expectedly positive for Del-1 (Supplementary Figure S1a). Moreover, we identified neuronal cells, characterized by NeuN staining, as a novel cellular source of Del-1 expression (Supplementary Figure S1b). In addition, the expression of Del-1 in neuronal cells was verified by using the human SH-SY5Y neuroblast cell line, which displayed strong Del-1 expression (Supplementary Figure S1c). Together, these data demonstrate for the first time that, besides endothelial cells, neuronal cells are a source for Del-1 in the CNS.

A previous whole genome association study implicated Del-1 as a candidate MS susceptibility gene[16]. We therefore investigated the regulation of Del-1 expression in EAE and MS. Quantitative PCR analysis demonstrated that Del-1 mRNA was downregulated in

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chronic active MS lesions, whereas Del-1 expression in chronic-inactive MS lesions was not significantly changed, as compared to samples from healthy control brain (Figure 1b). Consistently, Del-1 mRNA expression was decreased in the inflamed spinal cord of mice after induction of EAE (Figure 1c). Together, these data indicate that Del-1 expression is reduced in the course of human MS and murine EAE.

Del-1 deficiency increases EAE severity

Our findings so far suggested that the reduction of Del-1 expression may be associated with the development of neuroinflammatory and demyelinating disorders, such as MS or EAE. To address this hypothesis, we assessed the development of EAE in Del-1-deficient and -proficient mice. Strikingly, Del-1$^{-/-}$ mice displayed significantly increased EAE severity in the effector phase of the disease, as compared to WT mice (Figure 2a). Consistent with this finding, Del-1$^{-/-}$ mice showed higher extent of demyelination, as assessed by staining for myelin basic protein (MBP) (Figure 2b and c) and by luxol fast blue staining (Supplementary Figure S2). Moreover, Del-1$^{-/-}$ mice displayed increased axonal damage, as assessed by axonal staining with NF200 (Figure 2d), as compared to WT mice. Together, Del-1 deficiency increased EAE disease severity associated with increased demyelination and axonal damage.

Del-1 deficiency increases inflammation and immune cell infiltration in the CNS in the course of EAE

As the number of recruited inflammatory cells correlates with neuroinflammation and EAE severity[21, 22], and Del-1 is an endogenous negative regulator of leukocyte recruitment[10, 12], we next assessed inflammatory cell infiltration in the course of EAE. To analyze this in detail, we performed flow cytometric analysis, in order to study different immune cell populations in the inflamed spinal cord. As immune cell infiltration often precedes clinical disease development, we analyzed immune cell populations at the onset of the disease (defined as the first day of appearance of clinical symptoms; days 9-13)[23, 24] and in the effector phase (at the peak of the disease; day 19). Flow cytometric analysis was performed for CD4^{+} T cells, CD8^{+} T cells, neutrophils as well as recruited monocytes/macrophages. We found that Del-1$^{-/-}$ mice had significantly higher numbers of infiltrated neutrophils (defined as CD45^{+}F4/80^{-}Ly6G^{+}) at the onset and peak phase of the disease, whereas, strikingly, other cell populations, such as CD4^{+} or CD8^{+} cells or infiltrated monocytes/macrophages (defined as CD45^{high}CD11b^{+} cells)[25, 26] were not altered at the onset or peak of the disease due to Del-1-deficiency (Figure 3a-f).

Del-1 deficiency increases IL-17 levels and blood-brain barrier breakdown in the CNS in the course of EAE

We continued to assess possible mechanisms underlying the enhanced neutrophil infiltration in Del-1$^{-/-}$ mice. We and others have shown that IL-17 cytokine is capable of stimulating neutrophil accumulation in inflammation[15, 27, 28]. Moreover, as we have shown previously, IL-17 could decrease endothelial Del-1 expression *in vivo* and *in vitro*[15], whereas IL-17 levels were elevated in Del-1-deficiency[15]. We therefore assessed, whether IL-17 levels were upregulated in Del-1-deficiency in the course of EAE. IL-17 expression was increased

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in Del-1$^{-/-}$ mice (Figure 4a). In addition, more pro-inflammatory factors involved in EAE pathogenesis, such as IFN-γ, GM-CSF, TNF-α, IL-6 and iNOS, were upregulated in the inflamed spinal cord in Del-1-deficiency at the onset of EAE (Figure 4a and 4b). Furthermore, multiple pro-inflammatory mediators, such as the neutrophil-recruiting chemokine KC, the neutrophil granulocyte chemotactic receptor CXCR2, or further pro-inflammatory chemokines and cytokines, such as RANTES, MCP-1, IL-1β, TNF-α or IL-6, as well as iNOS and CCR2 were significantly increased in Del-1$^{-/-}$ mice as compared to WT mice at the peak of EAE disease (Supplementary Figure S3). Together, these findings suggest that increased EAE severity in Del-1 deficiency was associated with enhanced inflammation in the CNS.

We then set out to identify the source of the elevated IL-17 in the inflamed CNS due to Del-1-deficiency. As we found increased neutrophil infiltration in the inflamed spinal cord due to Del-1-deficiency and neutrophils have been implicated as a major effector cell type of the Th17 immune response, which is strongly implicated in EAE disease pathogenesis[28-30], we assessed whether the presence of IL-17-producing CD4^{+} cells (Th17 cells) was altered in Del-1-deficiency. To determine whether Th17 cells were altered in Del-1$^{-/-}$ mice upon EAE development, we isolated leukocytes from the inflamed spinal cord, re-activated them *in vitro* with MOG and then performed flow cytometric analysis for CD4 and IL-17. Unexpectedly, we found no increase in Th17 cells (defined as CD4^{+}IL-17^{+}) in the inflamed spinal cords of Del-1$^{-/-}$ mice (Figure 4c). Additionally, numbers of IFN-γ-producing CD4^{+} T cells (Th1) were also not altered by Del-1-deficiency at the onset of EAE (data not shown).

However, by this experimental approach, we identified increased numbers of IL-17-producing CD8^{+} T cells in the inflamed spinal cord owing to Del-1-deficiency (Figure 4d), although the total number of CD8^{+} T cells in the spinal cord was not enhanced in Del-1-deficient mice (Figure 3c and 3f). Interestingly, IL-17-producing CD8^{+} T cells have been recently identified as an important cellular player contributing to the pathogenesis of EAE and MS[31, 32]. To verify this finding by an independent approach, we performed histological immunofluorescence staining of Del-1-deficient and –proficient inflamed spinal cords for IL-17 and CD8. Consistently, we found significantly increased numbers of IL-17-positive CD8^{+} T cells in Del-1-deficient mice, as compared to control Del-1-proficient littermates (Supplementary Figure S4). In contrast, by flow cytometry and immunofluorescence staining we found that IL-17-production in myeloid cells[33] was not elevated in the inflamed spinal cord of Del-1-deficient mice (data not shown). Taken together, we found that CD8^{+} T cells (but not Th17 or myeloid cells) are the source of higher IL-17 levels in Del-1-deficiency.

We then assessed a further possible mechanism that could potentially account for enhanced neutrophil infiltration to the inflamed CNS in Del-1-deficiency. The breakdown of the BBB in EAE and MS promotes immune cell recruitment[34] and, several of the cytokines including IL-17 that we found upregulated in the Del-1$^{-/-}$ inflamed CNS have been implicated in BBB breakdown in EAE[34]. To assess permeability of the vessels of the inflamed CNS and, thus BBB breakdown, we studied the accumulation of systemically administered sodium fluorescein in the spinal cord. Del-1$^{-/-}$ mice displayed increased vascular permeability in

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the spinal cord upon EAE development, as compared to Del-1-proficient mice (Figure 4e). Taken together, both elevated cytokines, including IL-17, and enhanced BBB breakdown could account for the increased EAE severity and neutrophil accumulation into the inflamed spinal cord in Del-1-deficiency.

Abrogation of IL-17 signaling reverses the increased EAE severity due to Del-1-deficiency

Since we found increased IL-17 levels in the Del-1$^{-/-}$ inflamed CNS, we continued to identify whether the IL-17-dependent pathway was responsible for the increased EAE severity due to Del-1-deficiency. To this end, we generated Del-1$^{-/-}$IL-17RA$^{-/-}$ double deficient mice. Interestingly, the increased EAE disease severity due to Del-1-deficiency was reversed in Del-1$^{-/-}$IL-17RA$^{-/-}$ mice that developed a much milder EAE disease that was comparable to disease development in WT mice (Figure 5a); here it should be noted that independent experiments (e.g. as presented in Figure 2a or 5a) demonstrate the well-known variability in the time course and intensity of EAE development between experiments[35]. Moreover, Del-1$^{-/-}$ IL-17RA$^{-/-}$ mice showed decreased number of recruited neutrophils but no significant alterations in the numbers of CD4^{+} or CD8^{+} T lymphocytes, as compared to Del-1$^{-/-}$ mice (Figure 5b and 5c). Expression of another cardinal cytokine for EAE development, IFN-γ, was also increased in the inflamed spinal cords of Del-1$^{-/-}$ mice (Figure 4a). However, the EAE phenotype of Del-1-deficiency was unaffected by anti-IFN-γ treatment (data not shown). These data suggest that the increased EAE severity and inflammatory cell accumulation due to Del-1-deficiency is, at least in part, due to enhanced IL-17-mediated inflammation and neutrophil recruitment.

Administration of Del-1-Fc ameliorates EAE severity

Our findings so far demonstrated enhanced EAE-severity due to Del-1-deficiency. Given that Del-1 is a secreted molecule, one could envision that soluble Del-1 could represent a therapeutic approach in EAE. In a clinical setting of MS, therapeutic intervention can only be started after the onset of the disease, e.g., in an effort to inhibit disease relapse. We therefore engaged the relapsing-remitting EAE model in SJL/J mice and assessed the therapeutic efficacy of Del-1 administration therein. We have previously shown that Del-1-Fc fusion protein circulates and exerts anti-inflammatory actions[15]. Therefore, after the first clinical attack, mice were treated systemically with either Del-1-Fc or control-Fc. Strikingly, the clinical relapse was considerably ameliorated in mice receiving Del-1-Fc (Figure 5d), thus, suggesting that soluble Del-1 could serve as a novel therapeutic approach in MS.

Discussion

The present study identified for the first time that Del-1 is an endogenous homeostatic factor of the CNS protecting from neuroinflammation in the course of CNS autoimmunity. Del-1 limits the immune response and leukocyte recruitment to the inflamed CNS during EAE. Del-1$^{-/-}$ mice displayed exacerbated EAE disease severity with increased demyelination. Moreover, Del-1 expression was decreased in the inflamed CNS in murine EAE and in human chronic-active MS lesions. These novel findings, in combination with those by Goris *et al*. that identified Del-1 as a candidate MS susceptibility gene in a whole genome

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association study[16], suggest that Del-1 acts as an endogenous gatekeeper protecting from neuroinflammation.

IL-17 as well as IFN-γ have been strongly implicated in EAE development[29, 30, 36-38], although inhibition of IFN-γ-dependent signaling has yielded controversial results in EAE[39-41]. In this regard, Del-1-deficiency was associated with elevated IL-17 and IFN-γ, as well as with an increase in several pro-inflammatory cytokines. Whereas the increased EAE disease severity in Del-1-deficiency was reversible in Del-1$^{-/-}$IL-17RA$^{-/-}$ mice, blocking IFN-γ did not affect EAE severity in Del-1$^{-/-}$ mice. In keeping with the notion that IL-17-mediated inflammation stimulates neutrophil infiltration in the context of several diseases, including EAE[15, 27, 28], the increased severity of EAE due to Del-1-deficiency was accompanied by enhanced neutrophil accumulation, which was reversed in Del-1$^{-/-}$IL-17RA$^{-/-}$ mice. Moreover, we found increased BBB vascular permeability in Del-1$^{-/-}$ mice in the course of EAE; this finding could be related to the IL-17-mediated BBB breakdown, published previously[34, 42]. Our present findings agree with and substantiate the reciprocal regulatory loop between IL-17 and Del-1 that we have recently described to operate in inflammatory bone loss[15]. In particular, the IL-17-dependent downregulation of endothelial Del-1 promoted inflammatory cell recruitment and inflammatory bone loss, whereas Del-1-deficiency was associated with higher IL-17 levels and spontaneous inflammatory bone loss[15].

Interestingly, the major source of IL-17 upregulation in the inflamed spinal cord of Del-1$^{-/-}$ mice was CD8^{+} T cells, whereas Th17 cells did not seemingly contribute to elevated IL-17 in Del-1-deficiency. Despite the unabated association of Th17 cells with CNS autoimmunity[43, 44], the evidence that Th17 cells play a causative role in development of CNS autoimmunity is not as strong[44, 45]. The contribution of IL-17 isoforms and IL-17-receptor isoforms has been addressed in different studies. IL-17RA$^{-/-}$ or IL-17RC$^{-/-}$ mice develop a considerably ameliorated EAE disease[46, 47]. However, IL-17RA is expressed on several CNS cells, such as microglia or astrocytes[48], suggesting that IL-17, independent of its source, may contribute to CNS pathology. Our data presented here provide a paradigm where increased levels of IL-17 and enhanced IL-17-related immunopathology are not necessarily associated with elevated Th17 activity. Thus, caution is required when functions of the IL-17/IL-17R axis are interpreted; functions of this inflammatory axis do not always reflect Th17 activity; likely the actions of the IL-17/IL-17R axis are much broader than Th17 actions.

On the other hand, IL-17-producing CD8^{+} T cells have been identified in human MS lesions[49] and their contribution to the pathogenesis of murine EAE has been established recently[31]. We propose here that the absence of the endogenous homeostatic factor Del-1 results in higher inflammation with upregulation of several cytokines including IL-17, which derives from CD8^{+} T cells but not from Th17 cells, and that IL-17 can aggravate EAE by several mechanisms including enhanced neutrophil accumulation. Murine EAE models favor the induction of CD4^{+} T cell-triggered autoimmune mechanisms, whereas CD8^{+} T cells actually prevail in human MS lesions, which has been discussed as a potential reason for the frequent failure to translate therapeutic approaches from mice to men[32]. Del-1-deficient mice develop a CD8^{+}IL-17^{+} T cell-biased EAE; thus, EAE in Del-1-deficient mice

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may represent a more relevant mouse model for human MS, which merits further investigation.

Intriguingly, we show for the first time that besides endothelial cells, at least neuronal cells also express Del-1 in the CNS. Whether Del-1, besides its anti-inflammatory role to limit leukocyte infiltration, could directly regulate neuronal cell functions was not addressed in our present study and requires further investigation. Nevertheless, the previous identification of Del-1 as a potential MS susceptibility gene[16], together with our present findings that its levels are reduced in chronic active MS lesions in combination with the increased EAE development in Del-1$^{-/-}$ mice suggest that decline in the expression or function of Del-1 may be a predisposing factor for MS. We therefore propose Del-1 as an important endogenous component of the immune privilege status of the CNS. Interestingly, Del-1 was recently identified also as a disease susceptibility gene in Alzheimer's disease[50], suggesting that the protective function of Del-1 against neuroinflammation may be relevant in several CNS pathologies, which is worth assessing in future studies.

Finally, our findings suggest that Del-1 may provide a novel platform for developing effective therapeutic approaches for MS. As a proof of principle, we systemically treated EAE mice with Del-1-Fc, which we have previously shown to exert anti-inflammatory actions[15]. To better imitate the clinical setting, we chose the relapsing-remitting EAE model in SJL/J mice. Strikingly, Del-1-Fc administration after the first clinical attack resulted in a major amelioration of the EAE relapse. Although these data indicate that soluble Del-1-based therapeutic approaches may be promising for the treatment of EAE/MS, this possibility will have to be meticulously scrutinized in further preclinical models, for instance in non-human primates, before translating to a clinical scenario.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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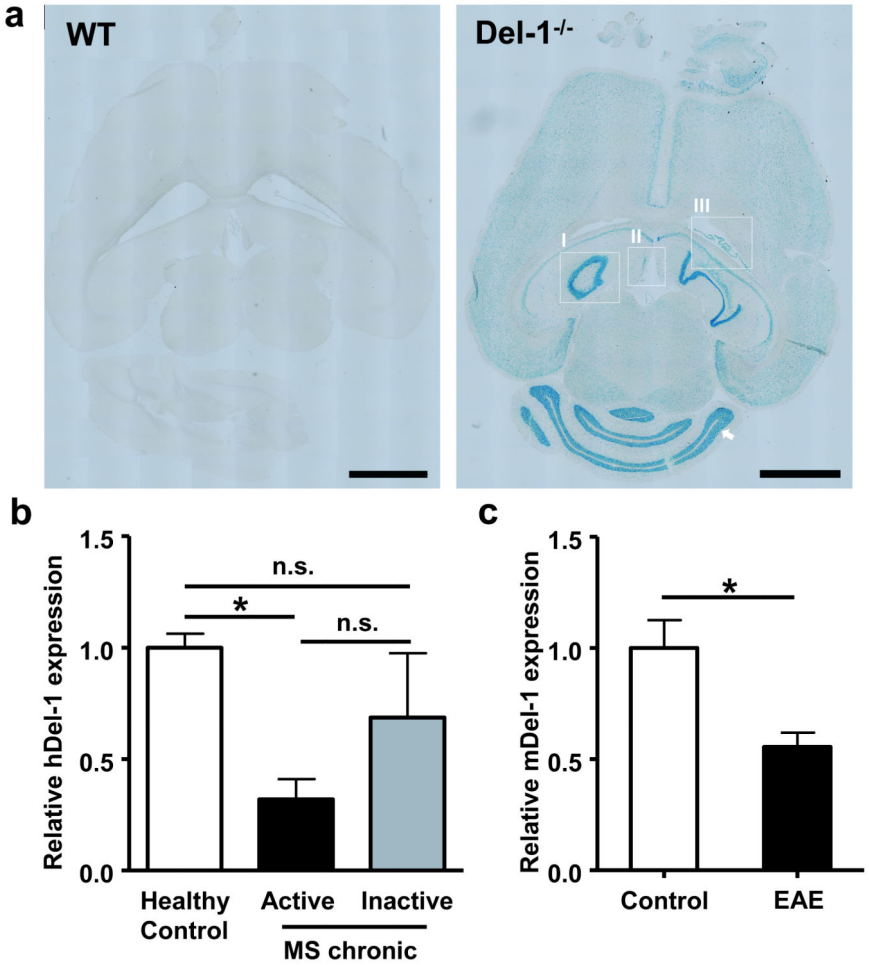

Figure 1. Del-1 is expressed in the CNS and down-regulated in neuroinflammation

(a) Del-1 is constitutively expressed in the CNS. Frozen sections obtained from 8-week old mice were subjected to staining for β-galactosidase to assess Del-1 expression. Staining of whole brain sections from a WT (left panel) and a Del-1$^{-/-}$ (right panel) mouse are shown. Del-1$^{-/-}$ mice contain a LacZ knock-in, whereby LacZ gene is controlled by the Del-1 promoter and thereby serves as a reporter for Del-1 expression. The white arrow indicates granular layers in cerebellum and I, II, III indicates hippocampus zone, subventricular zone, and choroid plexus, respectively. The stitched mosaic microscope images are shown. Scale bar = 2 mm. (b) Human Del-1 mRNA levels assessed in brain tissues from healthy controls or patients with multiple sclerosis (MS). The mRNA expression was normalized against 18S and the gene expression of white matter from healthy controls was set as 1. Data are means ± SEM (n = 5-6/group). (c) Mouse Del-1 mRNA levels assessed in the spinal cords from control or experimental autoimmune encephalomyelitis (EAE) mice on day 19 after MOG-immunization. 18S was used for normalization of mRNA expression and the gene expression of control mice was set as 1. Data are means ± SEM (n = 5-8 mice/group). * $P < 0.05$; n.s. not significant.

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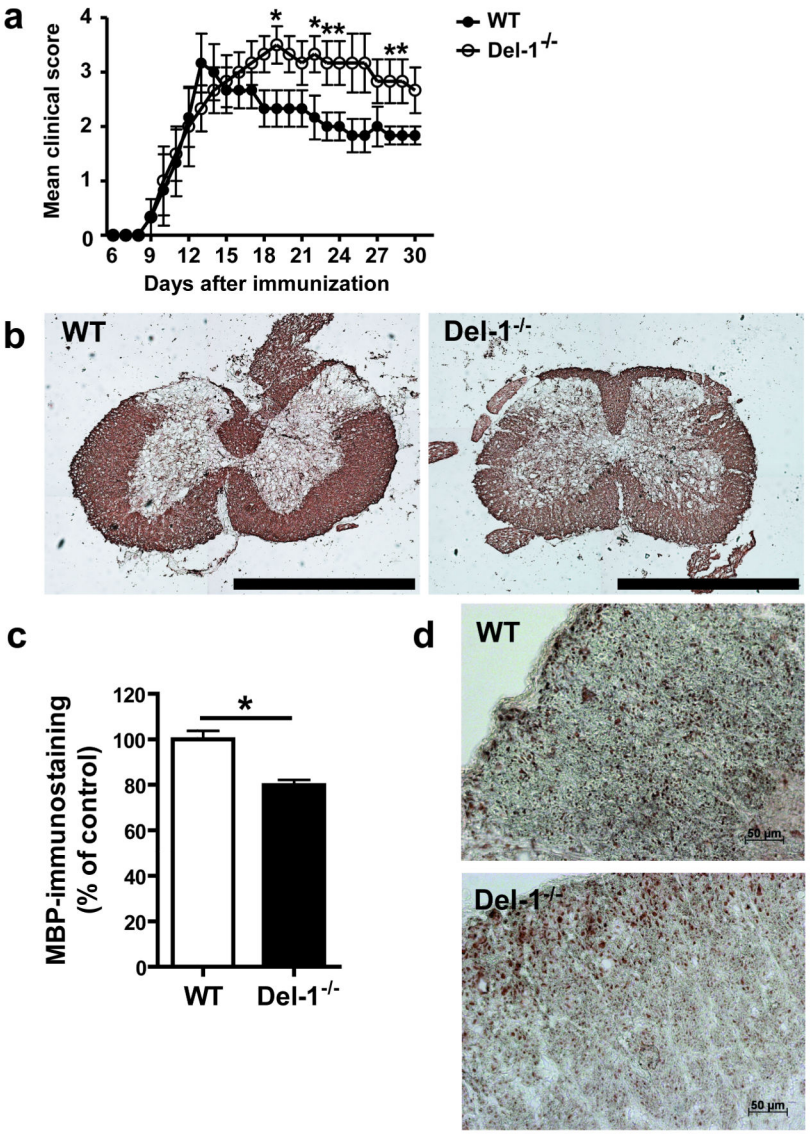

Figure 2. Del-1-deficiency aggravates EAE

(a) Clinical scores of WT and Del-1$^{-/-}$ mice after MOG-immunization. Data are means ± SEM (n = 6). A representative experiment is shown, similar results were observed in at least 3 additional independent experiments. (b-d) Myelin and axonal staining are shown in the spinal cords of WT and Del-1$^{-/-}$ mice on day 19 after MOG-immunization. (b) Sections were stained for MBP. The stitched mosaic microscope images are shown. Scale bar = 1 mm (c) The intensity of MBP immunostaining was assessed by ImageJ software; the intensity of MBP staining of WT mice represents the 100% control. Data are means ± SEM (n = 3 mice/group). (d) Sections were stained for neurofilament 200 to assess for neuronal damage. * $P < 0.05$.

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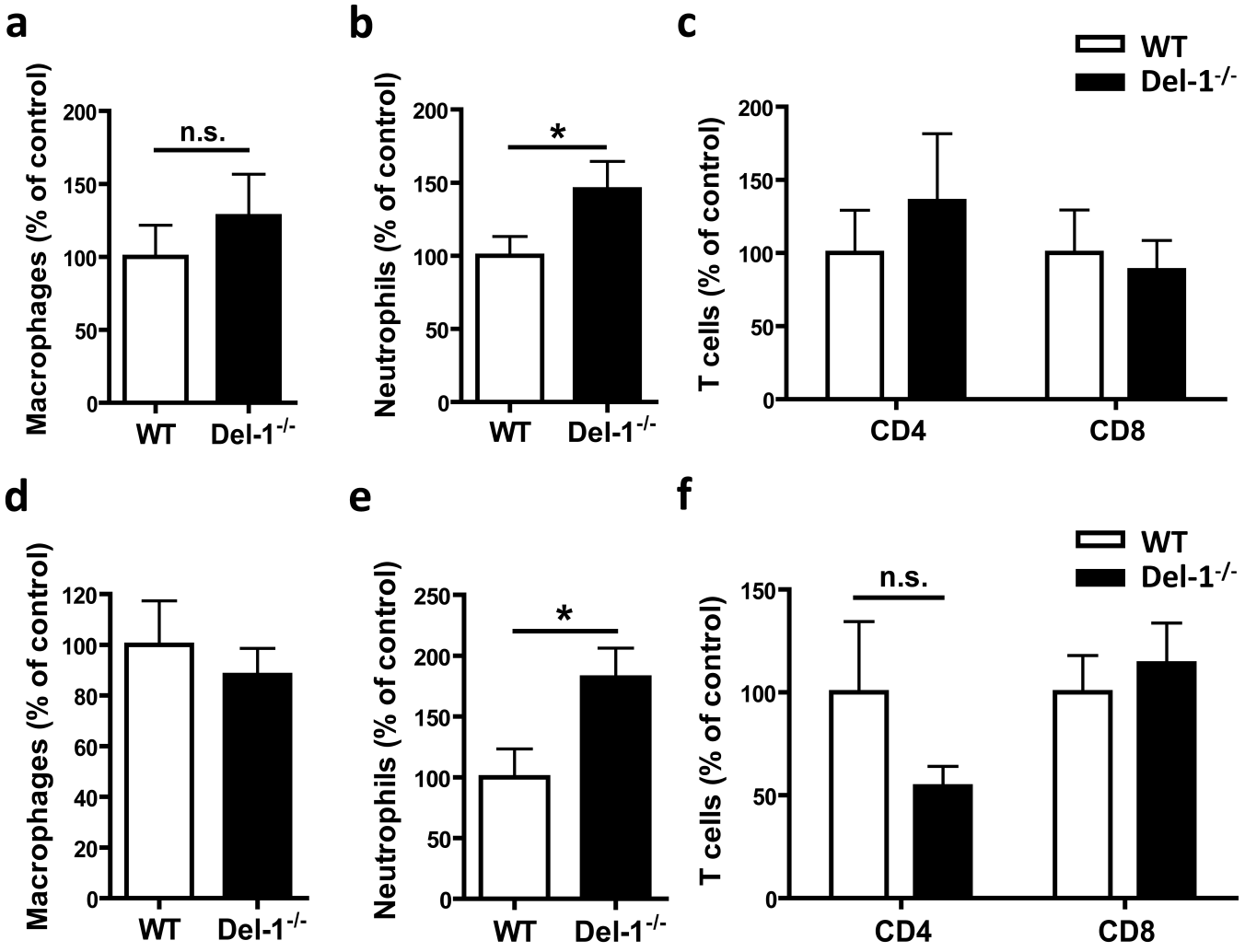

Figure 3. Del-1 deficiency is linked to increased recruitment of neutrophils to the inflamed spinal cord in the course of EAE

Leukocytes were isolated from the spinal cords of WT and Del-1$^{-/-}$ mice at the peak (a-c) or onset (d-f) of EAE disease. Flow cytometry analysis was performed for recruited monocytes/macrophages (CD45^{high}CD11b^{+}cells; a and d), neutrophils (CD45^{+}F4/80^{-}Ly6G^{+}; b and e), as well as CD4^{+} T cells and CD8^{+} T cells (c and f) at the peak (a-c; n = 15-17 mice/group) and onset (d-f; n = 8-10 mice/group) phases of EAE. The numbers of the respective cell types are shown as % of control; the absolute cell number of each cell type in WT mice was set as the 100% control. Data are means ± SEM. *$P < 0.05$; n.s. not significant.

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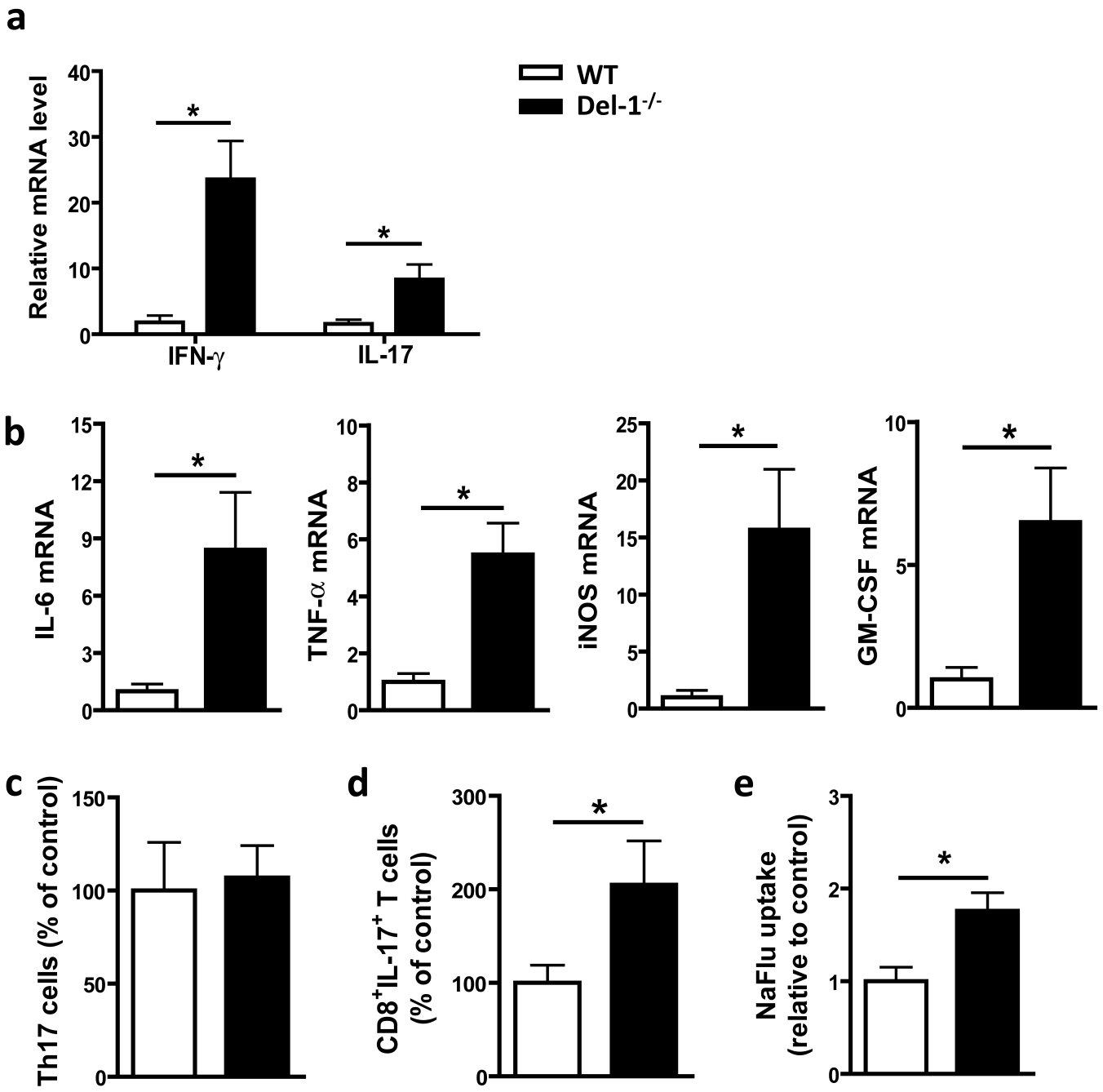

Figure 4. Increased IL-17 levels and accumulation of CD8$^+$IL-17$^+$ T cells as well as elevated blood brain barrier permeability owing to Del-1 deficiency in the course of EAE

(a, b) mRNA levels of (a) IFN-γ and IL-17 and (b) of further inflammatory mediators (IL-6, TNF-α, iNOS, and GM-CSF) are shown in the spinal cords of WT (open bars) and Del-1$^{-/-}$ (filled bars) mice at the onset of EAE. The mRNA expression was normalized against 18S and the gene expression of spinal cords of WT was set as 1. Data are means ± SEM (n = 6-8 mice/group). (c, d) Leukocytes were isolated from inflamed spinal cords at the peak of EAE, were re-stimulated with MOG *in vitro*, stained for intracellular IFN-γ and IL-17, together with CD4 and CD8 antibodies and then analyzed by flow cytometry. The numbers of the

respective cell types are shown as % of control; the absolute cell number of each cell type in WT mice was set as the 100% control. Data are means ± SEM (n = 13-15 mice/group). (e) Blood-brain barrier (BBB) permeability was assessed by NaFlu uptake in the spinal cords of WT and Del-1$^{-/-}$ mice at day 15 of EAE. The NaFlu uptake is shown; the NaFlu uptake of WT mice was set as 1. Data are means ± SEM (n = 6-7 mice/group). * $P < 0.05$.

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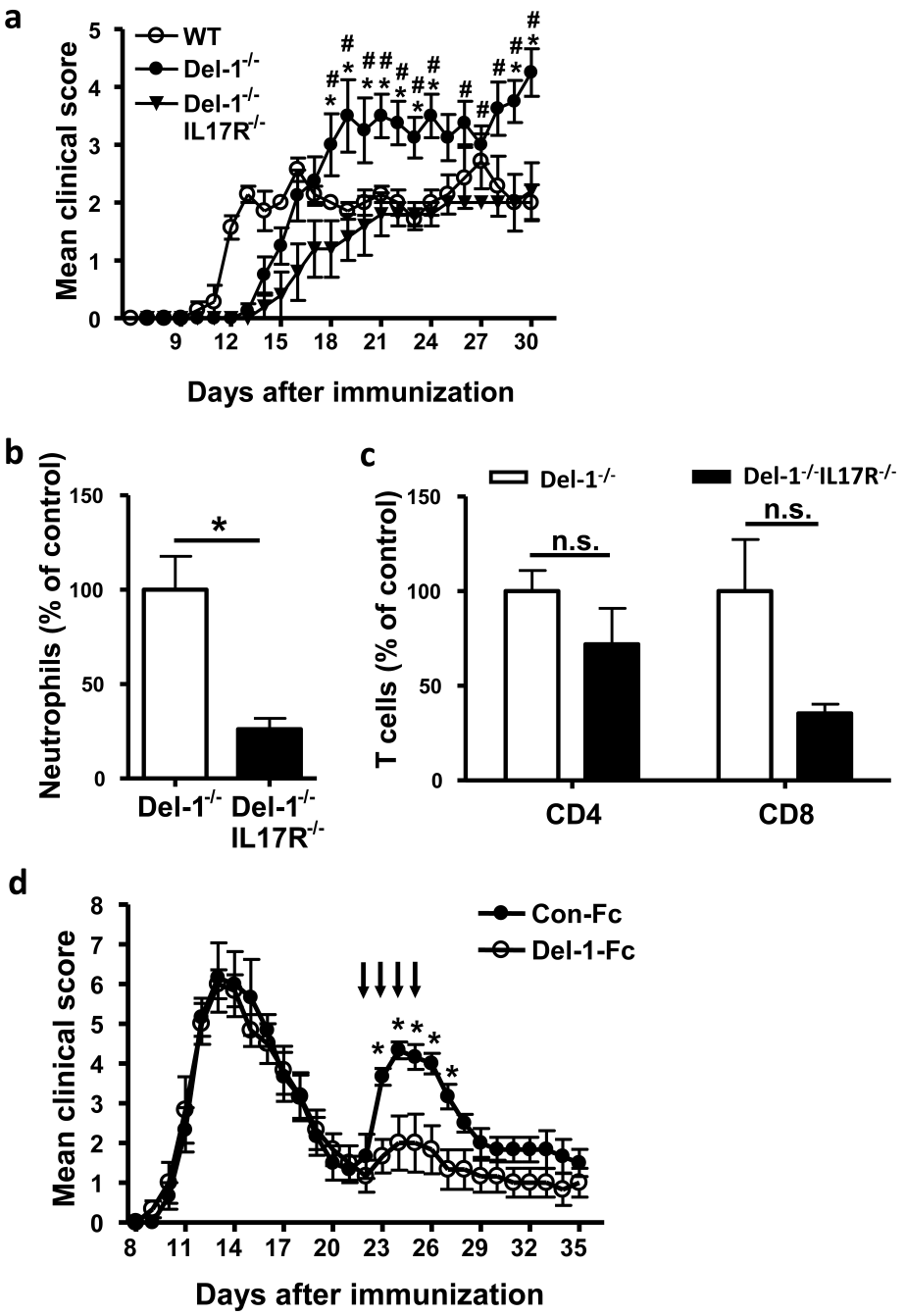

Figure 5. The phenotype of Del-1 deficiency in EAE is reversed by IL-17R deficiency and Del-1-Fc administration ameliorates relapsing-remitting EAE

(a) Clinical scores of WT, Del-1$^{-/-}$ and Del-1$^{-/-}$IL-17R$^{-/-}$ mice after MOG-immunization. Data are means ± SEM (n = 5-8). * and #: $P < 0.05$. *: indicates the statistical significance between Del-1$^{-/-}$ and WT mice; #: indicates the statistical significance between Del-1$^{-/-}$ and Del-1$^{-/-}$IL-17R$^{-/-}$ mice. (b-c) Leukocytes were isolated from the spinal cords of mice on day 19 after MOG-immunization and total leukocytes were counted and then analyzed by flow cytometry. Infiltrated neutrophils, defined as CD45^{+}F4/80^{-}Ly6G^{+}, as well as CD4^{+} T cells and CD8^{+} T cells are shown. The numbers of the respective cell types are shown as %

of control; the absolute cell number of each cell type in Del-1$^{-/-}$ mice was set as the 100% control. Data are means ± SEM (n = 3-8 mice/group). * $P < 0.05$; n.s. not significant. (d) Relapsing-remitting EAE was induced in SJL/J mice with PLP. Clinical scores of mice treated with Del-1-Fc or control-Fc for four consecutive days after the first clinical EAE attack. The arrows indicate the days of administration. Data are means ± SEM (n = 6 mice/group). * $P < 0.05$.

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