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Oligodendrocyte-Specific FADD Deletion Protects Mice from Autoimmune-Mediated Demyelination

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Apoptosis of oligodendrocytes (ODCs), the myelin-producing glial cells in the CNS, plays a central role in demyelinating diseases such as multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. To investigate the mechanism behind ODC apoptosis in EAE, we made use of conditional knockout mice lacking the adaptor protein FADD specifically in ODCs (FADDODC-KO). FADD mediates apoptosis by coupling death receptors with downstream caspase activation. In line with this, ODCs from FADDODC-KO mice were completely resistant to death receptor-induced apoptosis in vitro.

In the EAE model, FADDODC-KO mice followed an ameliorated clinical disease course in comparison with control littermates. Furthermore, these mice were almost completely protected against EAE induction when crossed to TNFR-1–deficient mice. However, it has been shown that the role of TNFR-1 in EAE pathogenesis is not restricted to its expression in ODCs; rather, this DR may also be important in mediating astrocytic, microglial, and endothelial responses as well as peripheral immune responses (11–16). Therefore, it is not clear whether the additional protective effect of TNFR-1 ablation is due to the lack of TNFR-1 signaling in ODCs or other cell types.

DRs are capable of inducing an apoptotic cell death program, involving the recruitment of procaspase-8 to the receptor complex leading to caspase-8 and downstream effector caspase activation (17). Crucial in this process is the receptor adaptor protein FADD, which bridges the receptor with procaspase-8 (18–21). Germline deletion of FADD in mice results in embryonic lethality as a result of cardiac failure and abdominal hemorrhage (22, 23). Fibroblasts from these mice are completely resistant to TNFR-1−/−, Fas−/−, and DR3−/− mediated apoptosis, demonstrating its crucial role in DR signaling (22). Besides its role in DR-induced apoptosis, FADD also appears to be important for T cell proliferation, since thymocytes form FADD−/−/RAG−/− chimeras or from mice expressing a T cell-specific dominant-negative FADD mutant display reduced proliferation in response to mitogens (24–26). Further studies showed that a C-terminal phosphorylation site of FADD is important for its function in inducing proliferation and cell cycle progression (27, 28).

In this study, we sought to address the role of FADD-dependent ODC apoptosis in the pathogenesis of EAE. Therefore, we gen-
generated mice lacking FADD specifically in ODCs (FADD<sup>ODC-KO</sup>). ODCs isolated from these mice were tested for their sensitivity to DR-induced apoptosis in vitro, and the response of FADD<sup>ODC-KO</sup> mice was analyzed in an EAE model. FADD-deficient ODCs were completely resistant to DR-induced apoptosis in vitro. FADD<sup>ODC-KO</sup> mice were significantly protected against EAE development, consistent with reduced immune cell infiltration, inflammation, and demyelination. Collectively, these data show that FADD is critical for ODC apoptosis, autoimmune inflammation, and demyelination in EAE, indicating that targeting FADD in ODCs might be an important option to combat MS and other demyelinating pathologies.

**Materials and Methods**

**Generation of ODC-specific FADD knockout mice**

To generate a conditional Fadd allele, we prepared a targeting vector to flank exon II of Fadd with two LoxP sites. An Frt site-flanked cassette, containing a neo gene, was placed into the second intron of the Fadd gene. A 4.0-kb fragment was used as 5′ homology region, a 2.5-kb fragment was placed between the two LoxP sites, and a 4.0-kb fragment was used as 3′ homology region. A thymidine kinase gene was used for negative selection homology region. A thymidine kinase gene was used for negative selection.

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containing the neomycin selection cassette (FADD<sup>FL/FL</sup>). The Frt-flanked neomycin cassette was removed by crossing FADD<sup>FL/FL</sup> mice with a Flp-deleter strain (30) generating a FADD floxed allele (FADD<sup>FL+</sup>). FADD<sup>FL/FL</sup> mice were crossed to MOG<sub>i</sub>-Cre (10) transgenic mice to generate ODC-<sup>−/−</sup> mice (FADD<sup>FL/FL</sup>-<sup>ODC-KO</sup>).

**Induction and assessment of EAE**

All experiments on mice were performed according to institutional, national, and European animal regulations. Mouse MOG peptide, aa 35–55 (MEVGWYRSPFSRVHLYRNGK), was synthesized by Sigma Genosys (Haverhill, U.K.). Ten- to 15-wk-old male mice received s.c. injection of 200 μg MOG peptide in 200 μl sterile PBS emulsified with an equal volume of CFA (Sigma Genosys) containing 5 mg/ml Mycobacterium tuberculosis H37Ra (BD Biosciences, Erembodegem, Belgium). Mice also received i.p. 50 ng pertussis toxin (Sigma Genosys, Cambridge, U.K.) in 200 μl sterile PBS, at the time of immunization and 48 h later. Clinical symptoms of disease were scored, as described before (12), on a scale of 0–6, with 0.5 points for immediate clinical findings as follows: 0, normal; 1, weakness of the tail; 2, complete loss of tail totality; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or 몽리; 6, death. To eliminate any diagnostic bias, mice were scored blindly.

**Histological analysis**

Mice were perfused by cardiac puncture of the left ventricle and postfixed with 4% paraformaldehyde. Spinal cords were dissected, dehydrated, and embedded in paraffin blocks. Sections of 2 μm were stained with H&E, Luxol fast blue (LFB) (Solvent Blue 38, practical grade; Sigma Genosys) for assessment of demyelination, and Abs against CD3 (Cetorox, Oxford, U.K.), MAC-3 (BD Biosciences), B220 (BD Biosciences), or amyloid precursor protein (APP) (Millipore, Watford, U.K.). Histological quantification was performed as described previously (31). Apoptosis in paraffin sections was visualized by TUNEL reaction according to the manufacturer’s instructions (Roche Diagnostics, Vilvoorde, Belgium). For NogoA staining, sagittal paraffin sections (5 μm thick) of formalin-fixed adult mouse brains were dewaxed in xylene, rehydrated, and incubated in 10 mM citrate buffer (pH 6) for 40 min at 94˚C. Nonspecific binding was blocked by incubation in 0.1% PBS containing 10% FCS and 1% Triton X-100 for 30 min. Sections were incubated overnight at 4˚C with mouse anti-NogoA (11C7, 1/20,000 in PBS; provided by D. Merkler). Olympus CellP software was used for image acquisition and cell counting. Only cells that were unequivocally NogoA positive were counted. Area sizes ranging from 300,000 to 700,000 μm<sup>2</sup>/brain area and animal were analyzed.

**Quantitative real-time PCR**

Total RNA was purified from spinal cord tissue 16 d postimmunization using TRIzol reagent (Invitrogen, Merelbeke, Belgium). RNA samples (1 μg) were treated with DNase I, Amp grade (Invitrogen) prior to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. cDNA (10 ng) was used for quantitative PCR in a total volume of 10 μl with LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and specific primers on a LightCycler 480 (Roche Diagnostics). Real-time PCR reactions were performed in triplicates. The following mouse-specific primers were used: TNF forward, 5'-ACCTCGG-TATGAGCCCATACAT-3'; TNF reverse, 5'-ACACCATCCCTCTCACA-GAGC-3'; IL-1β forward, 5'-CACCTACAAACGAGCAAGACAG-3'; IL-1β reverse, 5'-GCACTAGATACTCATGATATAC-3'; IFN-γ forward, 5'-GCCAAGCGGTACTGA-3'; IFN-γ reverse, 5'-TCAGTGAATTGAGTCAAGCTCATTACT-3'; IL-6 forward, 5'-GAGGATACATTCCACAGACAGC-3'; IL-6 reverse, 5'-AAATGTATCAGCTCTTCCACC-3'; MCP-1 forward, 5'-GCACTG-GGCCCTAAGGTCCTAT-3'; MCP-1 reverse, 5'-TGCTTGAGGTGGTTGGAGA-3'; RANTES forward, 5'-CGTCAGAAGTATTTATCTAC-3'; RANTES reverse, 5'-GGTCAAGAATACACCGACAC-3'; TGF-β forward, 5'-GCTGAACAGGAGACGCAAAAATG-3'; TGF-β reverse, 5'-GGATTTGTTAATCTTGTCTGTCAAGAC-3'; IL-10 forward, 5'-GTGACATCTAGC-GTACCT-3'; IL-10 reverse, 5'-GTGTTGTGAACTGTGCTaat-3'; Fasl forward, 5'-AATGTATCGCTCCTCTCACC-3'; Fasl reverse, 5'-CCACCTTCTTATTCTAC-3';

T cell recall assay

T cell recall responses were assessed in cells isolated from the spleen and CNS draining lymph nodes of mice 10 d after immunization with MOG peptide. After erythrocyte lysis using ACK lysis buffer, splenocytes and lymphocytes were cultured in flat-bottomed 96-well plates at a density of 7 × 10<sup>5</sup> cells/well in DMEM supplemented with 5% FCS, L-glutamine, 10 mM HEPES, and 100 U/ml penicillin/streptomycin at 37˚C in the presence of MOG<sub>35–55</sub> peptide at concentrations as indicated. After 48 h, cell cultures were restimulated with 0.5 μg/ml [3H]thymidine per well (1 μCi/ml [3H]thymidine deoxyribose); GE Healthcare, Diegem, Belgium) during the last 18 h. Cells were harvested onto glass fiber filter membranes by using a 96-well plate cell harvester (IHI110-96; Inotech, Dietikon, Switzerland), and thymidine incorporation was measured by scintillation counting (MicroBeta Plus 1450 reader; PerkinElmer, Zaventem, Belgium).

**Primary ODC cultures**

Brains were dissected from 0- to 2-d-old pups in cold PBS. After the removal of the meninges, the cortex was isolated and incubated in trypsin (0.25% trypsin in 1 mM EDTA) for 6 min at 37˚C. Cortices were washed

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**Table I. Clinical features of MOG-induced EAE in wild-type (FADD<sup>FL/FL</sup>) and FADD<sup>FL/ODC-KO</sup> mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence (%)</th>
<th>Mean Day of Disease Onset</th>
<th>Mean Maximal Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADD&lt;sup&gt;FL/FL&lt;/sup&gt; (n = 16)</td>
<td>16/16 (100)</td>
<td>14.3 ± 0.58</td>
<td>4.3 ± 0.24</td>
</tr>
<tr>
<td>FADD&lt;sup&gt;FL/ODC-KO&lt;/sup&gt; (n = 17)</td>
<td>13/17 (77)</td>
<td>15.5 ± 0.87</td>
<td>3.0 ± 0.33</td>
</tr>
</tbody>
</table>

Results are displayed as mean values ± SEM.

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**FIGURE 3.** Amelioration of EAE by targeting FADD in ODCs. A, EAE was induced in male FADD<sup>FL/ODC-KO</sup> mice (n = 17) and control (FADD<sup>FL/FL</sup>) littermates (n = 16). Clinical symptoms were scored and results are displayed as mean values ± SEM (representative of three different experiments; *p < 0.05). B, EAE was induced in male MOG<sub>i</sub>Cre/+ transgenic mice (n = 5) and control wild-type (WT) littermates (n = 5). Clinical symptoms were scored and results are displayed as mean values ± SEM.
sections from the lumbar spinal cord were examined for infiltrating rTNF (10^5 IU/ml) or FasL/CD95L-Fc (gift from Dr. Martin Leverkus, Sigma Genosys), 0.1% BSA, D-(+)-galactose, and antibiotics. Cells were centrifuged at 1100 rpm for 6 min. Cells were resuspended in DMEM supplemented with 10% FCS, l-glutamine, sodium pyruvate, and antibiotics, after which the tissues were triturated by pipetting twice in DMEM supplemented with 10% FCS, l-glutamine, sodium pyruvate, and antibiotics, after which the tissues were triturated by pipetting twice in DMEM supplemented with 5 μg/ml poly-L-lysine (Sigma Genosys) and incubated at 37°C and 5% CO₂. Medium was replaced after 2 d of incubation and every 2–4 d thereafter. At days 9–12 of incubation, microglia were removed by orbital shaking of the flasks at 150 rpm for 1 h. Fresh culture medium was added and flasks were shaken vigorously 20 times. Medium was removed and centrifuged at 1100 rpm for 6 min. Cells were resuspended in DMEM supplemented with GlutaMAX, N1 medium supplement (Sigma Genosys), ascorbic acid, 6,7-dimethyl-5,6,7,8-tetrahydropterine (Sigma Genosys), glutathione, triiodo-L-thyronine sodium salt (Sigma Genosys), insulin (Sigma Genosys), 0.1% BSA, d-(+)-galactose, and antibiotics. Cells were counted and cultured in poly-L-lysine-coated 6-well or 12-well plates at a density of 2 × 10^5 cells/cm². Purified ODCs were treated with mouse rTNF (10^6 IU/ml) or FasL/Cd95L-Fc (gift from Dr. Martin Leverkus, Mannheim, Germany) in cell culture medium and visualized by phase contrast light microscopy.

Western blot analysis

Protein lysates were prepared from purified ODCs, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting. Membranes were probed with anti-FADD, anti-caspase-8 (gift of Andreas Strasser and Lorraine O’Reilly, Walter and Eliza Hall Institute, Melbourne, Australia), and anti-actin (Santa Cruz Biotechnology, Heidelberg, Germany) Abs.

Electron microscopy

Mice were perfused with 5 U/ml heparin in cold PBS by cardiac puncture of the left ventricle followed by perfusion with a sodium phosphate buffer containing 1% paraformaldehyde and 1% glutaraldehyde. Brains and spinal cord were dissected and kept in fixative overnight, after which the corpus callosum was dissected. Mouse corpus callosum and spinal cord were washed twice for 30 min in sodium cacodylate buffer (0.1 M [pH 7.3]; Sigma Genosys) embedded in agarose (Sigma Genosys type VII, catalog no. A-4018), and sectioned at 500 μm and placed in PBS. Corpus callosum and spinal cord were dissected out and osmicated for 1 h at room temperature in 2% OsO₄ in sodium cacodylate buffer. Samples were dehydrated through a graded ethanol series, followed by embedding in Spurr’s resin. Ultrathin sections of a gold interference color were cut using an ultramicrotome (Leica EM UC6), followed by poststaining with uranyl acetate for 40 min and for 7 min in lead citrate in a Leica EM AC20 and collected on formvar-coated copper slot grids. They were viewed with a transmission electron microscope JEOL 1010 (JEOL, Tokyo, Japan). Electron micrographs were analyzed for fiber diameter, axon diameter, and myelin thickness, and G ratios were defined as diameter of the axon divided by fiber diameter (axon plus myelin). We calculated a G ratio for each fiber and subsequently averaged all G ratios from one brain.

Statistical analysis

Results are expressed as the means ± SEM. Statistical analysis between experimental groups was assessed using an unpaired two-sample Student’s t test.

**Results**

**Generation of mice deficient of FADD in ODCs**

We generated mice carrying a conditional Fadd allele in which exon II of the mouse Fadd gene, encoding the death domain of FADD, is flanked by LoxP sites (Fig. 1A). This conditional Fadd allele allows the tissue-specific and/or inducible inactivation of FADD through expression of Cre recombinase. Mice homozygous for the LoxP-flanked Fadd allele (Fadd^FL/FL) express normal levels of FADD and develop normally. Deletion of the LoxP-flanked Fadd allele (Fadd^FL/FL) in all cells of the body. FADD^+/− mice, generated by crossing heterozygous FADD^+/− mice, did not express FADD (Fig. 1B) and died during embryogenesis (data not shown), as expected from the embryonic lethal phenotype of the published FADD knockout mice (22, 23).

**FIGURE 4.** Reduced inflammation and demyelination in the CNS of FADD^DC-KO mice. Histological profiles of spinal cords from FADD^DC-KO mice and control (FADD^FL/FL) littermates 25 d after EAE induction. A. Sections from the lumbar spinal cord were examined for infiltrating macrophages (MAC3, brown), T cells (CD3, brown), and B cells (B220, brown) by immunohistochemistry. B. Assessment of demyelination by LFB (blue) staining and (C) axonal damage by APP (brown) immunohistochemistry. Scale bar, 100 μm. Original magnification ×10.

**FIGURE 5.** Reduced apoptosis in FADD^DC-KO mice during EAE. A. TUNEL staining on spinal cord section of FADD^DC-KO mice and control (FADD^FL/FL) littermates 16 d after EAE induction. Scale bar, 20 μm. Original magnification ×40. B. Quantification of the number of TUNEL-positive cells/section from FADD^DC-KO mice (n = 4) and control (FADD^FL/FL) littermates (n = 8). Error bars represent SEM. *p < 0.01.

<table>
<thead>
<tr>
<th></th>
<th>MAC3/mm²</th>
<th>CD3/mm²</th>
<th>B220/mm²</th>
<th>Demyelination (%)</th>
</tr>
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<tbody>
<tr>
<td>FADD^FL/FL (n = 7)</td>
<td>102.4 ± 17.2</td>
<td>50.3 ± 8.0</td>
<td>14.6 ± 1.9</td>
<td>33.6 ± 3.2</td>
</tr>
<tr>
<td>FADD^DC-KO (n = 7)</td>
<td>22.2 ± 5.9</td>
<td>10.9 ± 3.5</td>
<td>2.7 ± 0.8</td>
<td>7.4 ± 2.3</td>
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</tbody>
</table>

Values are means ± SEM. For all parameters, p < 0.05.

Numbers of infiltrating T cells (CD3), B cells (B220), and macrophages (MAC3) and percentage demyelination in FADD^DC-KO mice and control wild-type littermates (FADD^FL/FL) 25 d after EAE induction. Results are displayed as mean values ± SEM. For all parameters, p < 0.05.
Mice lacking FADD specifically in ODCs were generated by crossing FADD<sup>FL/FL</sup> mice to transgenic mice expressing the Cre recombinase under control of the ODC-specific MOG (MOGi-Cre) promoter (10). ODC-specific FADD knockout (FADD<sup>FL/FL/ODC-KO</sup>) mice reached adulthood without any evidence of CNS defects. Histological examination of the CNS of FIGURE 6. Reduced proinflammatory gene expression in CNS of FADD<sup>ODC-KO</sup> mice. Quantitative measurements of the indicated cytokine and chemokine mRNA expression in spinal cord from FADD<sup>ODC-KO</sup> (n = 3) and control (FADD<sup>FL/FL</sup>) littermate (n = 3) mice 16 d after immunization are shown. For all cytokines and chemokines tested, \( p < 0.05 \).

![Image](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)

FIGURE 7. Peripheral T cell activation is unaltered in FADD<sup>ODC-KO</sup> mice. Splenocytes and lymphocytes from CNS-draining lymph nodes from MOG peptide-immunized control (FADD<sup>FL/FL</sup>) and FADD<sup>ODC-KO</sup> mice were cultured and stimulated with the indicated concentrations of MOG peptide. A, T cell proliferation was assessed by measurement of [\(^{3}\text{H}\)]thymidine incorporation by liquid scintillation counting. Results are expressed as cpm of triplicate cultures. B, Culture supernatants were collected 48 h after MOG peptide stimulation and assayed for IL-2, IFN-\( \gamma \), and IL-17 by ELISA. Upper panels show ELISA on spleen lymphocyte cultures; lower panels show ELISA on lymph node lymphocyte cultures. Results are shown as means ± SEM.
adult FADD\(^{ODC-KO}\) mice (25 wk of age) did not show any sign of dysmyelination or demyelination. Brains from FADD\(^{ODC-KO}\) mice showed normal myelin distribution and no signs of altered myelination on the light microscopical level (data not shown). Electron microscopical examinations showed comparable numbers of myelinated axons with structurally intact myelin sheath and regular thickness between FADD\(^{ODC-KO}\) and wild-type control mice (Fig. 1C).

To verify the tissue specificity of the ODC-specific FADD knockout, we performed PCR on DNA isolated from various tissues of a FADD\(^{ODC-KO}\) mouse, which showed the deleted knockout allele only in CNS tissue (total brain and spinal cord), while recombination was not detected in all other tested tissues (Fig. 1D). Additionally, immunoblot analysis on protein extracts from primary ODC cultures derived from FADD\(^{ODC-KO}\) and control mice revealed efficient ablation of FADD in ODCs from FADD\(^{ODC-KO}\) mice (Fig. 1E). Finally, we performed NogoA stainings on brain sections from FADD\(^{ODC-KO}\) mice and control littermates and counted the number of NogoA-positive cells. As shown in Supplemental Fig. 1, both groups of mice have similar numbers of ODCs in the different brain areas analyzed.

ODCs from FADD\(^{ODC-KO}\) mice are resistant to DR-mediated cell death in vitro

ODCs express members of the TNFR superfamily, including TNFR-1 and Fas/CD95 (7, 33), and receptor stimulation with TNF or FasL/CD95L, respectively, is known to induce apoptotic cell death in vitro (4, 7, 9, 34). To examine the effect of FADD deletion in ODCs on their sensitivity to DR-induced apoptosis, primary ODCs from FADD\(^{ODC-KO}\) mice and control littermates were isolated and stimulated in vitro with TNF. ODCs from FADD\(^{ODC-KO}\) mice were completely resistant to TNF, in contrast to control ODCs, which progressively died in time (Fig. 2A, 2B). A similar protection was observed in ODCs from FADD\(^{ODC-KO}\) mice stimulated with FasL (Fig. 2C). This clearly demonstrates the importance of FADD in DR-induced apoptosis signaling in ODCs, suggesting a potential role for FADD in ODC apoptosis as part of the pathogenesis of EAE and MS.

Amelioration of EAE by targeting FADD in ODCs

To study the ODC-specific function of FADD-dependent signaling in the pathogenesis of EAE, we immunized FADD\(^{ODC-KO}\) and control littermates, carrying the LoxP-flanked allele but lacking the expression of the Cre recombinase (FADD\(^{FL/FL}\) which served as wild-type controls in all experiments reported in this study), with MOG\(_{35-55}\) and monitored disease progression by clinical assessment. Both immunized FADD\(^{ODC-KO}\) and control littermate mice developed EAE symptoms (loss of tail tonicity and hind limb paralysis) starting at approximately day 15, indicating that the “priming” phase of the disease is unaffected (Table I). Control mice followed a typical disease course and developed signs of severe paralysis, with an incidence of 100% and reaching a mean maximal clinical score of 4.3 (Fig. 3A, Table I). In contrast, 23% of FADD\(^{ODC-KO}\) mice did not develop clinical scores >1.5, and they developed a considerably less severe disease, showing only mild paralysis and reaching a mean maximal clinical score of 3.0 (Fig. 3A, Table I). Furthermore, to control for a possible influence of the MOGi-Cre transgene, we immunized MOGi-Cre transgenic mice and transgene-negative littermates with MOG\(_{35-55}\) and found no differences between both groups (Fig. 3B), suggesting that expression of the MOGi-Cre transgene in the absence of the LoxP-flanked Fadd allele does not affect the course of EAE. Taken together, these results indicate that FADD-dependent signaling in ODCs has a pathological function in EAE.

Milder histopathological insult in FADD\(^{ODC-KO}\) mice

To investigate the reason for the improved clinical outcome in FADD\(^{ODC-KO}\) mice, histological analysis was performed on spinal cord sections from FADD\(^{ODC-KO}\) and control littermates 25 d after immunization. In control mice, large numbers of MAC3+ macrophages, CD3+ T cells, and B220+ B cells were detected in the meninges and in the spinal cord parenchyma (Fig. 4A), accompanied by histological signs of severe demyelination in the white matter as shown by LFB staining (Fig. 4B), as well as signs of axonal damage as visualized by immunohistochemical staining for APP (Fig. 4C). In contrast, in spinal cords from FADD\(^{ODC-KO}\) mice the numbers of inflammatory cells were substantially reduced, whereas myelin remained largely intact and only few signs of axonal damage could be detected (Fig. 4, Table II), confirming the clinical observation that these mice are mostly protected from EAE. These results demonstrate that loss of FADD in ODCs protects mice from EAE pathology by impairing immune cell infiltration in spinal cord parenchyma.

Reduced ODC apoptosis in FADD\(^{ODC-KO}\) mice during EAE

Multiple studies showed that demyelination accompanies the loss of ODCs in late MS lesions (35, 36) and in EAE lesions induced by MOG peptide immunization (4, 8). Apoptotic ODCs in regions surrounding MS/EAE plaques support the idea that ODC apoptosis is tightly associated with the pathology of the disease and may be partly responsible for the progression of EAE (3). To study apoptosis in situ during EAE, spinal cords from FADD\(^{ODC-KO}\) mice and control littermates were isolated on day 16 after EAE induction and analyzed for apoptosis by TUNEL staining. Numerous TUNEL-positive cells were seen in the lesions from control mice 16 d after immunization, presumably representing dying ODCs and inflammatory cells. FADD\(^{ODC-KO}\) spinal cord, however, showed much fewer TUNEL-positive cells (Fig. 5). These results demonstrate reduced apoptosis in FADD\(^{ODC-KO}\) mice and suggest that a defective activation of the apoptotic signaling cascade in ODCs could account for the protection from EAE in FADD\(^{ODC-KO}\) mice.

FIGURE 8. Model for progression of EAE in FADD\(^{FL/FL}\) and FADD\(^{ODC-KO}\) mice. A first wave of T cells is activated by immunization with MOG peptide (1) in the periphery, causing inflammation in the CNS after crossing a disrupted blood–brain barrier (BBB). In FADD\(^{FL/FL}\) mice, this inflammation causes ODC cell death (2). Debris derived from these dying cells may act as secondary epitopes, allowing for the activation of a second wave of T cells that further contribute to EAE progression. In FADD\(^{ODC-KO}\) mice, ODC cell death is inhibited, resulting in a lack of secondary epitopes (3). As a consequence, the initial CNS inflammation is not amplified and EAE disease stabilizes.
Reduced proinflammatory gene expression in spinal cord of FADDODC-KO mice

To examine whether ODC-specific FADD deficiency affects the expression of proinflammatory genes during EAE, we performed quantitative real-time PCR analysis on spinal cord tissue to determine the expression of several cytokines and chemokines that have been implicated in EAE pathogenesis. All cytokines and chemokines tested were strongly upregulated in the CNS of control mice at day 16 postimmunization, whereas their induction was significantly reduced in FADDODC-KO mice (Fig. 6). These findings strongly suggest that inhibition of FADD-dependent signaling in ODCs leads to a decreased expression of key mediators involved in CNS inflammation.

Peripheral T cell activation is unaltered in FADDODC-KO mice

Although FADDODC-KO mice show a significant reduction in EAE severity, they still show initial clinical signs of disease pathology, suggesting that the initial inflammation, imposed by peripheral Ag-specific T cell activation and expansion followed by blood–brain barrier infiltration, is not affected in FADDODC-KO mice. To confirm this, we analyzed the generation of MOG35-55-specific T cells in FADDODC-KO mice. Splenocytes and lymphocytes from CNS-draining lymph nodes from FADDODC-KO and control mice were isolated 10 d postimmunization, after which their in vitro response to secondary exposure to MOG35-55 peptide was analyzed. T cell responses to secondary MOG35-55 stimulation were comparable between FADDODC-KO and control mice, as assessed by proliferation and IL-2, IL-17, and IFN-γ production (Fig. 7). These results demonstrate that peripheral T cell activation is unaltered in FADDODC-KO mice.

Discussion

This study demonstrates that FADD-mediated cell death of ODCs, the myelin-producing glial cells of the CNS, is a critical pathological event in the progression and development of EAE. The decreased clinical response in FADDODC-KO mice correlates with improved myelin integrity, a reduction in lymphocyte and macrophage infiltration into the spinal cord parenchyma, and a reduction in inflammatory cytokine and chemokine expression. These observations indicate that FADD-dependent DR signaling in ODCs is crucial to ODC damage, myelin breakdown, and associated neurologic symptoms. FADD mediates apoptotic signaling from multiple DRs, including TNFR-1 and Fas. This is also the case in ODCs, as illustrated by our finding that primary ODCs from FADDODC-KO mice are completely resistant to in vitro TNF stimulation. Because TNFR-1 and Fas have previously been suggested to contribute to ODC apoptosis in AEE and MS (10), it can be expected that the observed protection in FADDODC-KO mice in this study at least partially reflects defective TNFR-1 and Fas signaling in these cells. Besides Fas- and TNFR-1–dependent mechanisms, other DR and non-DR–dependent mechanisms may mediate ODC injury in MS (37). For example, nerve growth factor, which is increased in cerebrospinal fluid of MS patients, has the ability to trigger ODC death via the p75 neurotrophin receptor, a DR belonging to the TNFR superfamily (38, 39). Interestingly, the mechanism of apoptosis by nerve growth factor through the p75 neurotrophin receptor is different from TNFR and Fas-mediated killing and does not involve FADD or caspase-8 activation (40). ODC apoptosis and myelin damage can also be initiated upon triggering of glutamate and ATP receptors (41, 42). Finally, semaphorin 4D, an axonal guidance molecule released by activated T cells present in demyelinating lesions of the spinal cord from patients suffering from neuroinflammatory demyelination, is able to induce caspase-dependent ODC apoptosis, most likely through receptors of the plexin family (43).

Although ODC death is suppressed and FADDODC-KO mice show a significant reduction in EAE severity, they still show initial clinical signs of disease pathology. Because we showed that peripheral T cell activation is unaltered in FADDODC-KO mice, CNS-resident cells, including microglia and astrocytes, can be expected to still become activated and to express several proinflammatory cytokines and chemokines that act in concert to establish a favorable environment for infiltrating encephalitogenic T lymphocytes (12, 44). However, we propose that further disease progression in FADDODC-KO mice is suppressed since ODCs are protected against the cytotoxic activity of specific death ligands, such as TNF and FasL. Additionally, because less myelin debris will be produced in FADDODC-KO mice, a phenomenon termed “epitope spreading” is limited. All initial activated autoreactive T lymphocytes show a TCR specificity for the MOG epitope that was used to immunize mice. As the disease progresses, however, encephalitogenic T lymphocytes show a diversification in their auto-Ag specificity to subdominant epitopes of the disease-inducing Ag and even to epitopes distinct from the initial Ag (45). As such, progression of EAE and MS involves a shifting of T cell autoreactivity from primary initiating self-determinants to defined cascades of secondary determinants that sustain the inflammatory self-recognition process during disease progression (46). This process has been reported in MS patients (47) as well as in EAE (48–51) and is presumed to contribute to the chronic progression of the disease (45). As the production of myelin debris is indissolubly associated with epitope spreading, this might provide a plausible additional mechanism by which ODC-specific FADD ablation leads to the preservation of myelin and the amelioration in EAE pathology in FADDODC-KO mice (Fig. 8). Moreover, these data suggest that myelin debris might activate innate-mediated inflammation in the brain that is needed for the development of full-blown inflammation, a process that is suppressed in FADDODC-KO mice.

In conclusion, our data implicate FADD as a critical mediator of ODC apoptosis, which is tightly associated with destruction of the myelin sheath, leading to axonal dysfunction and degeneration. Moreover, our data strongly suggest that ODC apoptosis is needed to initiate and sustain inflammation, and that FADD-dependent ODC apoptosis is critical for disease development. Most importantly, our finding that ODC-specific deletion of FADD significantly reduces clinical symptoms, demyelination, and CNS inflammation in EAE suggests that targeting FADD might provide an effective therapeutic strategy in MS and other demyelinating pathologies of the CNS.

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Disclosures

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References


