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CD30 ligand is a new therapeutic target for central nervous system autoimmunity

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21

22 **Abstract**

23 The CD30 ligand (CD30L)/CD30 axis plays a critical role in Th1 and Th17 cell differentiation.
24 However, the role in the pathogenesis of central nervous system autoimmunity remains unknown.
25 Here we show the resistance for experimental autoimmune encephalomyelitis (EAE) with markedly
26 reduced induction of antigen-specific Th1 and Th17 cells in CD30L knockout mice. Bone marrow
27 (BM) chimera experiments indicated that CD30L on BM-derived cells were critical for the
28 development of EAE and that CD30L reverse signaling in CD4 T cells was dispensable for the
29 pathogenic Th17 cell differentiation at the induction phase. Adoptive transfer experiment revealed an
30 additional role for CD30L in the environment at the effector phase. *In vivo* neutralization of CD30L by
31 soluble murine CD30-Immunoglobulin fusion protein before disease onset or even after disease onset
32 significantly ameliorated the clinical symptoms. These results indicate that CD30L/CD30 signaling is
33 critically involved in antigen-specific CD4 T cell responses at both the induction and effector phase,
34 thus could be a new target molecule for the treatment of central nervous system autoimmunity.

35

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS, which is induced actively by immunization with myelin antigen (Ag) or passively by adoptive transfer of myelin-specific CD4 T cells into naive mice [1, 2]. Both Th1 and Th17 cells were shown to induce EAE, as Th1 cells might promote the entry of Th17 to the CNS during EAE [3, 4]. We have recently reported that tyrosine kinase 2 (Tyk2), which is involved in interleukin (IL)-12-signaling for Th1 cell differentiation and IL-23-signaling for Th17 cell differentiation, plays an indispensable role in the development of EAE [5]. Thus, both IL-12/Th1 and IL-23/Th17 axis might be involved in the pathogenesis of EAE.

CD30 ligand (CD30L, CD153, TNFSF8) is a 40-kDa type II membrane-associated glycoprotein belonging to the tumor necrosis factor (TNF) superfamily (TNFSF) [6], and is expressed on macrophages, dendrocytes (DCs), $CD4^+CD3^-CD11c^-$ accessory cells, B cells, activated CD4 T cells, and $\gamma\delta$ T cells [7-10]. CD30 (TNFRSF8), the receptor for CD30L and a member of the TNF receptor super family (TNFRSF), is a 120 kDa type I membrane associated glycoprotein and preferentially expressed on activated or memory helper T cells but not on resting cells [11, 12]. CD30 binds several TNF receptor-associated factors (TRAFs) and activates nuclear factor κ B (NF- κ B) mainly via

TRAF2 [13, 14]. As for other TNFSF/TNFRSF members, reverse signaling via CD30L has been reported [7, 15, 16]. In the past, CD30L/CD30 signaling was thought to be preferentially involved in Th2 cell responses [17-19]. However, we have revealed that CD30L/CD30 axis was involved in both Th1 and Th2 cell responses [20-22], and regulatory T cells were reported to suppress allograft rejection via a CD30-dependent mechanism [23, 24]. Furthermore, we recently found that the CD30L/CD30 axis had a critical role in Th17 differentiation *in vitro* and *in vivo* [16, 25]. Thus, CD30L/CD30 signaling may not be linked to a commitment step for a particular subset differentiation, but might promote cell survival of pre-activated T cells, resulting in an acceleration of various helper T cell responses.

In the present study, we found that CD30L knockout (KO) mice were resistant to myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide-induced EAE. The numbers of MOG₃₅₋₅₅ peptide-specific Th1 and Th17 cells in the draining lymph nodes (dLNs) were markedly reduced in CD30L KO mice as compared with wild type (WT) mice. CD30L/CD30 signaling on CD4 T cells promoted Ag-specific CD4 T cell responses at the induction phase, and CD30L in the environment was important at the effector phase in mice developing EAE. *In vivo* neutralization of CD30L by soluble murine CD30-immunoglobulin (mCD30-Ig) fusion protein significantly ameliorated EAE when administered at either the induction or effector phase in WT mice. These results indicated that

70 the CD30L/CD30 axis is critically involved in pathogenic T cell responses not only in dLNs at the
71 induction phase but also in spinal cords at the effector phase, and thus modulation of CD30L/CD30
72 signaling could be a new biological therapy for the treatment of MS and other inflammatory CNS
73 demyelinating diseases.

74

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Japan KBT (Shizuoka, Japan). CD30L KO mice were generated as previously described [26, 27]. All mice were maintained under specific-pathogen free conditions at our institute and were backcrossed with C57BL/6 mice at least eight times. All mice were used at 8–12 weeks of age. All experiments were approved by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University.

2.2. Antibodies and reagents

MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) peptide was purchased from Medical & Biological Laboratories (Nagoya, Japan). Fcγ receptor blocking monoclonal antibody (mAb) (anti-CD16/32, 2.4G2), allophycocyanin (APC) and APC-H7-conjugated anti-CD4 (RM4-5), APC-conjugated CD45.2 (104), FITC-conjugated anti-CD45.1 (A20), V450 and FITC-conjugated anti-CD11b (M1/70), FITC-conjugated anti-Foxp3 (FJK-16s), PerCP-Cy5.5-conjugated anti-major histocompatibility complex (MHC) class II (M5/144.15.2), PE-conjugated anti-CD30 (CD30.1), PE-conjugated anti-CD30L (RM153), PE-conjugated anti-CD154 (MR1), PE-conjugated anti-Gr-1 (RB6-8C5), PE-Cy7-conjugated anti-IFN-γ (XMG1.2), Alexa Flour 647-conjugated anti-CC chemokine receptor (CCR) 6 (140706) mAbs were purchased from BD Biosciences (San Diego, CA). Alexa Flour

647-conjugated anti-CD3 (17A2), Alexa Flour 647-conjugated IL-17A (TC11-18H10) mAb, rat IgG2b was purchased from e-Bioscience (San Diego, CA). Recombinant mouse IL-23 was purchased from R&D Systems (Minneapolis, MN).

2.3. Active EAE

Female mice at 8–12 weeks of age were subcutaneously (s.c.) immunized with 200 µg of MOG₃₅₋₅₅ peptide emulsified with complete Freund's adjuvant (CFA) containing 500 µg of *Mycobacterium tuberculosis* H37RA (BD Biosciences) on day 0 and were i.p. injected with 500 µg of pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) on day 0 and 2 [1]. Clinical symptoms were scored according to the following criteria: 0, unaffected; 1, flaccid tail; 2, impaired gait; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, total hind limb paralysis with partial forelimb paralysis [28].

2.4. Passive EAE

Donor mice were s.c. immunized with 200 µg of MOG₃₅₋₅₅ peptide emulsified with CFA. Ten days later, dLNs were harvested and single-cell suspensions were incubated in the presence of 10 µg/ml of MOG₃₅₋₅₅ peptide and 10 ng/ml of recombinant IL-23 in a 37°C, 5% CO₂ incubator for 72 hours. Recipient mice were irradiated with 4 Gy and intravenously transferred with 2×10^7 cultured cells on day 0 and i.p. injected with 500 µg of PTX on day 0 and 2 [2].

109 *2.5. Thymidine incorporation assay*

110 Whole dLNs cells were cultured in 96-well plates with 10 µg/ml of MOG₃₅₋₅₅ peptide for 72 hours.

111 Wells were pulsed with ³H-thymidine (New England Nuclear, Boston, MA) at 37 mBq/well for the

112 final 12 hours. Mean incorporation was measured by a 1205 Betaplate scintillation counter

113 (PerkinElmer, Gaithersburg, MD).

114 *2.6. Isolation of mononuclear cells from spinal cords*

115 Spinal cords were excised from mice perfused transcardially with ice-cold phosphate buffered saline

116 (PBS). They were cut into small pieces and digested with collagenase D (Invitrogen, Carlsbad, CA)

117 and DNase I (DN25; Sigma-Aldrich, Tokyo, Japan). Dispersed cells were passed through a nylon

118 mesh, placed onto 33% Percoll solution (GE Healthcare, Buckinghamshire, UK), and centrifuged at

119 800 ×g for 20 minutes. Cell pellets were suspended with culture medium and used for analysis.

120 *2.7. Flow cytometric analysis and intracellular staining*

121 For cell surface staining, single-cell suspensions were incubated with an optimal concentration of

122 fluorescent Abs in Hanks' Balanced Salt Solution with 0.5% fetal bovine serum for 20 minutes at 4°C.

123 Intracellular staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences) according

124 to the manufacturer's instructions. In some experiments, we added propidium iodide (1 µg/ml) to cell

125 suspensions just before running on a flow cytometer to detect and exclude dead cells for the analysis.

Stained cells were run on a FACS Calibur or FACS Verse flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

2.8. Measurement of MOG₃₅₋₅₅ peptide-specific cytokine production

Single cell suspensions were harvested from dLNs of EAE induced mice at day 9. Whole dLNs cells were cultured in 96-well plates with 10 µg/ml of MOG₃₅₋₅₅ peptide for 72 hours. The amount of cytokines in the culture supernatants was measured by DuoSet ELISA Development System (R&D Systems). For intracellular staining, single-cell suspension from dLNs or spinal cords were incubated for 5 hours with 25 µg/ml of MOG₃₅₋₅₅ peptide in the presence of 10 µg/ml of brefeldin A (Sigma-Aldrich) for the last 4 hours.

2.9. Histopathological analysis

Mice were transcardially perfused with PBS and 4% paraformaldehyde in 0.1 M PBS. Spinal cords were excised and embedded in optimal cutting temperature compound and sectioned horizontally on a cryostat. Sections were stained by hematoxinilin and eosin (H&E) and Klüver-Barrera's staining.

2.10. Generation of bone marrow chimera

Bone marrow (BM) cells were extracted from WT (Ly5.1/5.1) and CD30L KO (Ly5.2/5.2) mice by flushing the femurs and tibias and were then depleted of T cells using anti-CD3 mAb (17A2, eBioscience) and Dynabeads sheep anti-rat IgG (Invitrogen). Red blood cells were lysed with 0.83%

ammonium chloride. Remaining cells were intravenously injected into lethally (10 Gy) irradiated recipient mice. For mixed BM chimeras, BM cells from WT (Ly5.1/5.1) and CD30L KO (Ly5.2/5.2) were mixed at a ratio of 1:1 and were intravenously injected into lethally irradiated recipient mice (Ly5.1/5.2). Eight weeks later, BM reconstitution was confirmed and EAE was induced.

2.11. Generation and in vivo administration of mCD30-Ig

A soluble mCD30-Ig fusion protein was obtained as previously described [25, 29]. mCD30-Ig fusion protein cDNA was expressed by the vector pBMGNeo in NIH 3T3 cells. Secreted mCD30-Ig protein from growing NIH 3T3 cells in serum-free medium (SFM101; Nissui Pharmaceutical, Tokyo, Japan) was purified by HiTrap Protein G HP (GE Healthcare) and analyzed by SDS-PAGE for purity. mCD30-Ig, diluted at a concentration of 1 mg/mL in PBS, was stored -80°C until use. For *in vivo* neutralization, 200 μg of mCD30-Ig or PBS was i.p. injected into recipient mice.

2.12. Statistical analysis

Statistical significance was evaluated by Student's *t*-test and Dunnett's test using JMP9 software (SAS, Cary, NC). Differences with *p* values < 0.05 were considered statistically significant.

3. Results

3.1. *CD30L KO mice are resistant to active EAE.*

We examined the role of the CD30L/CD30 axis in the pathogenesis of active EAE induced by immunization of mice with MOG₃₅₋₅₅ peptide emulsified with CFA, followed by intraperitoneal (i.p.) injection with PTX on days 0 and 2. WT mice started to develop EAE about 10 days after immunization and reached a peak score at about 3 weeks with 100% incidence, whereas clinical symptoms were significantly ameliorated in CD30L KO mice when compared with WT mice (Fig. 1A). Histological analysis of spinal cords was performed 18 days and 40 days after immunization for EAE induction. Inflammatory cell infiltration was reduced in CD30L KO mice compared with WT mice at the peak disease phase (Fig. 1B), and the severity of demyelination was attenuated in CD30L KO mice at the chronic phase (Fig. 1C). To quantify the extent of cellular infiltration, flow cytometric analysis was performed and revealed significantly impaired infiltration of CD4⁺MHC Class II⁻ (CD4 T cell), CD11b⁺MHC Class II⁺ (macrophage/microglia) and Gr-1⁺MHC Class II⁻ (granulocyte) cells in CD30L KO mice (Fig. 1D). Thus, the CD30L/CD30 axis is involved in the development of active EAE.

3.2. *CD30L/ CD30 signaling is involved in MOG₃₅₋₅₅ peptide-specific Th1 and Th17 responses at the*

175 *induction phase in mice after immunization*

176 To examine the role of the CD30L/CD30 axis in the development of MOG₃₅₋₅₅ peptide-specific CD4 T
177 cells, dLNs were harvested 9 days after immunization and were restimulated *in vitro* with or without
178 various concentrations of MOG₃₅₋₅₅ peptide. Ag-specific T cell proliferation was assessed by
179 thymidine incorporation assay. Thymidine uptake was significantly reduced in CD30L KO mice
180 compared with WT mice (Fig. 2A). Ag-specific T cells are reported to be defined by intracellular
181 staining of CD154 after *in vitro* stimulation with Ag [30]. As shown in Fig. 2B, the frequency and
182 number of CD154⁺ CD4 T cells in dLNs was significantly lower in CD30L KO mice compared with
183 WT mice. Furthermore, intracellular cytokine staining of a cell population gated on CD4⁺ T cells after
184 restimulation with MOG₃₅₋₅₅ peptide revealed that CD30L KO mice had significantly reduced levels of
185 MOG₃₅₋₅₅ peptide-specific Th1 and Th17 cells during the development of EAE (Fig. 2C). However,
186 there were no differences in the percentages and cell numbers of Forkhead box p3 (Foxp3)⁺ regulatory
187 CD4 T cells in dLNs between WT and CD30L KO mice (Fig. S1). We further examined cytokine
188 production from dLNs cells harvested on day 9 after immunization by enzyme-linked immunosorbent
189 assay (ELISA) after 3 days culture in the presence of MOG₃₅₋₅₅ peptide. Consistent with flow
190 cytometric analysis, dLNs cells from CD30L KO mice produced significantly attenuated levels of
191 IL-17 and interferon (IFN)- γ (Fig. 2D), whereas there were no differences in IL-2, IL-4, IL-10 and

192 TNF- α production between WT and CD30L KO mice (data not shown).

193 We previously reported that CD30⁺T-CD30L⁺T cell interactions mediated Th1 cell responses in

194 *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) infection, and played a critical role in Th17

195 cell differentiation *in vitro*, while CD30L expression on Ag-presenting cells (APCs) was dispensable

196 in the development of Ag-specific Th1 and Th17 cells [16, 21]. To determine which cells are involved

197 in mice developing EAE, we examined the expression pattern of CD30L. As previously described[8],

198 CD30L was expressed on CD4 T cells, CD8 T cells, macrophages and DCs (Fig. S2). Unique

199 CD4⁺CD3⁻CD11c⁻ accessory cells were reported to be the constitutive source of CD30L [9]. However,

200 we could not detect its expression. Subsequently, we cultured dLNs cells in a 96-well plate containing

201 medium with MOG₃₅₋₅₅ peptide (10 μ g/ml), or in a plate coated by anti-CD3 mAb (5 μ g/ml) for 24

202 hours, and examined CD30L expression. CD30L expression was enhanced by stimulation with

203 MOG₃₅₋₅₅ peptide, and almost all CD4 T cells expressed CD30L after stimulation by anti-CD3 mAb

204 (Fig. S3). To address the possibility that CD30L on APCs is important for Ag presentation to CD4 T

205 cells, we purified CD4 T cells from dLNs on day 9 after immunization and co-cultured them with 30

206 Gy-irradiated splenocytes from WT and CD30L KO mice as APCs in the presence of MOG₃₅₋₅₅

207 peptide for 72 hours. There were no differences in IL-17 and IFN- γ production in the culture

208 supernatant as measured by ELISA between WT and CD30L KO APCs (Fig. 2E). Thus, CD30L

expression on APCs is dispensable during Ag presentation to primed CD4 T cells to induce IL-17 and IFN- γ production.

Furthermore, we also examined the expression levels of CCR6 on CD4 T cells at the induction phase, and found that the frequency and cell number of CCR6⁺IL-17A⁺ CD4 T cells were significantly attenuated in CD30L KO mice compared with WT mice (Fig. S4). Importantly, there were no differences in the percentages of CCR6⁺ cells in Th17 cell between WT and CD30L KO mice. Thus, we considered that CCR6 expression was diminished in association with impaired development of Th17 cells in CD30L KO mice.

3.3. CD30L on hematopoietic cells and radioresistant cells was involved in EAE development

As previously described, CD30L was expressed on various cell types including activated CD4⁺ helper T cells, macrophages, DCs in dLNs at the induction phase (Fig. S2). We also found an appreciable level of CD30L expression on macrophages and microglial cells in addition to infiltrating CD4 T cells in the spinal cord at the effector phase (Fig. S5). To elucidate the role of CD30L expression on BM derived cells or radioresistant host cells in EAE development, we set up three groups of BM chimeras (Fig. 3A): (1) Recipient WT (Ly5.2/5.2) mice were lethally irradiated and transferred with BM cells from WT (Ly5.1/5.1) mice (WT $\rightarrow$ WT); (2) Recipient CD30L KO (Ly5.2/5.2) mice were lethally

226 irradiated and transferred with BM cells from WT (Ly5.1/5.1) mice (WT -> CD30L KO); and (3)
227 Recipient WT (Ly5.1/5.1) mice were lethally irradiated and transferred with BM cells from CD30L
228 KO (Ly5.2/5.2) mice (CD30L KO -> WT). After confirming BM reconstitution 8 weeks later, BM
229 chimera mice were immunized with MOG₃₅₋₅₅ peptide emulsified with CFA. As shown in Fig. 3B and
230 C, development of EAE in CD30L KO->WT mice were significantly ameliorated compared with WT
231 -> WT mice, confirming that CD30L expression on BM derived cells have a pivotal role in EAE
232 development. Although the severity of EAE was comparable to WT -> WT mice, it was notable that
233 the onset of EAE was significantly delayed in WT -> CD30L KO mice. These results suggest that
234 CD30L expression on BM derived cells has a critical role but its expression on radioresistant host cells
235 might at least partly be involved in EAE development.

236

237 *3.4. CD30L reverse signaling in CD4 T cells is dispensable for their differentiation into Ag-specific*

238 *CD4 T cells*

239 We previously reported that both CD30 and CD30L are expressed on activated helper T cells and that
240 both CD30 signaling and CD30L reverse signaling in CD4 T cells are involved in Th17 differentiation
241 [16]. To determine whether CD30L reverse signaling in CD4 T cells is involved in the development of
242 EAE, we next performed a mixed BM chimera experiment. As shown in Fig. S6A, recipient WT mice

243 (Ly5.1/5.2) were lethally irradiated and transferred with a mixture of BM cells from WT (Ly5.1/5.1)
 244 and CD30L KO (Ly5.2/5.2) mice. After confirming reconstitution 8 weeks after transfer, the chimera
 245 mice were immunized with MOG₃₅₋₅₅ peptide emulsified with CFA. The mixed BM chimera mice
 246 developed mild EAE, probably due to the effect of artificial manipulation by bone marrow
 247 transplantation and the relatively old week-age recipient mice (Fig. S6B). On day 10 after
 248 immunization, dLNs cells were harvested and the expression of CD154, intracellular staining of
 249 IL-17A and IFN- γ in CD4 T cells were examined after restimulation with MOG₃₅₋₅₅ peptide for 5 hours.
 250 The frequencies of CD154⁺CD4 T cells, IL-17A⁺CD4 T cells and IFN- γ ⁺ CD4 T cells in
 251 CD30L-deficient CD4 T cells were comparable to those in WT CD4 T cells (Fig. S6C and D). Twenty
 252 days after immunization, we examined the chimerism of CD4 T cells from dLNs and spinal cords. The
 253 ratio of WT (Ly5.1/5.1) to CD30L KO (Ly5.2/5.2) CD4 T cells was similar between spinal
 254 cord-infiltrating cells and dLNs cells (Fig. S6E). These results indicate that reverse signaling via
 255 CD30L on CD4 T cells is dispensable for their differentiation into MOG₃₅₋₅₅ peptide-specific CD4 T
 256 cells and infiltration into the spinal cord.

257

258 *3.5. CD30L in the environment at the effector phase is involved in EAE development and accumulation*
 259 *of spinal cord inflammatory cells*

We detected CD30L expression in macrophage and microglia cells in addition to activated CD4 T cells in the spinal cord of WT mice developing EAE (Fig. S5) and BM chimera experiments suggested that CD30L expression on radioresistant host cells might at least partly be involved in the development of EAE (Fig. 3B and C). To examine the requirement of CD30L in the environment, we next induced passive EAE by transferring MOG₃₅₋₅₅ peptide-primed WT CD4 T cells into naive WT and CD30L KO mice. Naive CD30L KO recipients developed significantly attenuated EAE and significantly mild body weight loss after receiving MOG₃₅₋₅₅ peptide-primed WT CD4 T cells (Fig. 4A and B). Consistent with these results, histopathological examination performed 40 days after transfer revealed reduced infiltration of mononuclear cells into spinal cords by H&E staining (Fig. 4C) and reduced demyelination in Klüver-Barrera's staining in CD30L KO mice (Fig. 4D). These results indicate that CD30L in the environment, possibly on microglia, also plays an important role in the recruitment and/or *in situ* activation of effector CD4 T cells in the spinal cord during the effector phase of EAE.

3.6. Soluble mCD30-Ig-treatment inhibits EAE development

We examined the effect of inhibition of CD30L/CD30 signaling by soluble mCD30-Ig fusion protein in the course of active EAE in WT mice (Fig. 5A). Clinical scores and body weight loss were significantly improved in mice treated with mCD30-Ig at the induction phase (Fig. 5B and C).

277 Histological analysis performed on day 18 showed reduced infiltration of mononuclear cells into CNS
278 parenchyma (Fig. 5D). The frequency of MOG₃₅₋₅₅ peptide-specific CD4 T cells examined by CD154
279 staining and MOG₃₅₋₅₅ peptide-specific Th17 cells were significantly decreased in dLNs of mice
280 treated with mCD30-Ig at the induction phase (Fig. 5E and F). These results indicated that the
281 administration of soluble mCD30-Ig at the induction phase inhibited Ag-specific CD4 T cell responses
282 and ameliorated active EAE.

283 Our study revealed that the CD30L/CD30 axis was involved in the development of EAE both at the
284 induction phase in dLNs and at the effector phase in the spinal cord after EAE induction. Therefore,
285 we examined the effect of mCD30-Ig administered at the effector phase after disease onset (Fig. 6A).
286 Mice administered mCD30-Ig on days 12, 14, 16, 18 and 20 after disease onset had reduced clinical
287 scores and body weight loss at the chronic phase (Fig. 6B and C). Consistent with clinical symptoms,
288 histopathological analysis performed on day 40 revealed reduced infiltration of mononuclear cells and
289 apparently reduced demyelination in the spinal cords of mCD30-Ig-treated mice compared with
290 controls (Fig. 6D and E). Thus, *in vivo* blockade of CD30L/CD30 signaling by soluble mCD30-Ig
291 even after onset of EAE significantly protected WT mice against EAE, suggesting the therapeutic
292 application of CD30-Ig for MS treatment.

4. Discussion

This study elucidated a modulatory role for CD30L/CD30 signaling in the pathogenesis of EAE. First, we observed that CD30L KO mice were resistant to active EAE. At the induction phase, CD30L/CD30 signaling, presumably executed by T-T interactions, was involved in the differentiation of MOG₃₅₋₅₅ peptide-specific Th1 and Th17 cells in dLNs. At the effector phase in spinal cord, CD30L was expressed on macrophages and microglia as well as infiltrating CD4 T cells. Furthermore, MOG₃₅₋₅₅ peptide primed T cells induced milder passive EAE in CD30L KO mice than that in WT mice, suggesting that CD30L in the environment at the effector phase is also involved in the development of EAE. *In vivo* neutralization of CD30L by soluble mCD30-Ig fusion protein ameliorated EAE in WT mice when administered before and after disease onset. These results indicate that the CD30L/CD30 axis plays an important role in the two-step activation of CD4 T cells during the pathogenesis of EAE, and thus could be a new target molecule for the treatment of MS.

We previously reported that CD30L/CD30 signaling executed by CD30⁺T-CD30L⁺T cell interactions is involved in Th1 responses that produce IFN- γ against BCG infection [21]. Furthermore, we recently found that naïve CD4⁺CD44^{low}CD62^{hi} T cells purified from CD30 KO or CD30L KO mice with C57BL/6 background exhibited impaired Th17 cell differentiation after *in vitro* culture under Th17-polarizing conditions [16]. In the present study, we consistently found that priming of MOG₃₅₋₅₅

310 peptide-specific Th1 and Th17 cells in dLNs was greatly reduced in CD30L KO mice compared with
311 WT mice at the induction phase of EAE, and that there was no difference in recall responses of
312 WT CD4 T cells using WT or CD30L KO APCs.

313 Similar to other members of TNFSF proteins [31], it was reported that CD30L might transmit reverse
314 signals to downregulate IL-2 production in CD4 T cells [7, 15, 16]. Because IL-2 is reported to inhibit
315 Th17 differentiation [32], CD30L reverse signaling on CD4 T cells might be also responsible at least
316 in part for differentiation of Th17 cells via down-regulation of IL-2 production. However, IL-2
317 production by CD30L-deficient CD4 T cells in response to MOG₃₅₋₅₅ peptide was not significantly
318 increased in CD30L KO mice. Furthermore, mixed BM chimera experiments revealed that reverse
319 signaling was dispensable at the induction phase in mice developing EAE.

320 After priming in dLNs, effector CD4 Th1 cells producing IFN- γ and Th17 cells producing IL-17A
321 leave dLNs and traffic through the choroid plexus into the subarachnoid space, where they encounter
322 Ag presented by meningeal APCs such as resident microglia and inflammatory DCs/macrophages/B
323 cells [33]. Consequently, the effector helper T cells are restimulated and undergo clonal expansion,
324 producing cytokines that evoke EAE. In the present study, adoptive transfer experiments revealed that
325 CD30L expression on host CNS cells was required at the effector phase in the CNS. Interestingly, BM
326 chimera experiments revealed that expression of CD30L on BM-derived donor cells played a critical

327 role in the development of EAE, and that CD30L on radioresistant host CNS cells, presumably
328 resident microglia, also participated in the development of EAE, especially at early stages of the
329 effector phase. CD30L is expressed on various cells including activated CD4 T cells, macrophages,
330 DCs, CD4⁺CD3⁻CD11c⁻ accessory cells, B cells, and $\gamma\delta$ T cells [7-10]. We also detected CD30L
331 expression on microglia cells in addition to infiltrating activated CD4 T cells in the spinal cord of WT
332 mice developing EAE, during which processing and presentation of myelin Ag in the CNS is required
333 for initiation of disease progression [34]. Activated CD4 T cells interacts MHC Class II⁺ APCs and are
334 reactivated in the subarachnoid space [35], and reactivation of autoreactive T cells is reported to
335 determine the severity of EAE [36]. The impaired recruitment and/or *in situ* reactivation of effector
336 CD4 T cells in the spinal cord at the effector phase might be partly responsible for the disease
337 resistance observed in CD30L KO mice.

338 Brain lesions in mice developing EAE preferentially express C-C Chemokine receptor (CCR) 6⁺
339 effector T cells recruited by C-C chemokine ligand (CCL) 20 [37]. This raises an alternative
340 possibility that CD30L/CD30 signaling recruits effector T cell subsets to the CNS via expression of
341 chemokines on the blood brain barrier and/or chemokine receptors on T cells. As shown in Fig. S4, our
342 present results indicated that the numbers of CCR6⁺CD4 T cells were significantly reduced in CD30L
343 KO mice in association with impaired development of Th17 cells. However, the Th17 cells in CD30L

344 KO mice expressed the equivalent level of CCR6 to those in WT mice. CD30L/CD30 signaling was
345 reported to upregulate CCR7 rather than CCR6 in human YT lymphoma cell line [39]. Furthermore, it
346 was also reported to upregulate the expression of CCL21, a ligand for CCR7 in the absence of
347 lymphotoxin α [40]. Th17 cells are reported to express CCR6 preferentially [37]. Therefore, the
348 reduced numbers of CCR6⁺CD4 T cells in CD30L KO mice may be simply as a result of impaired
349 Th17 response in CD30L KO mice and not due to defect in direct signaling via CD30L/CD30
350 signaling. Further study should be required to clarify the possibility.

351 We previously reported that both Th1 and Th17 responses in lamina propria of the intestine were
352 impaired in CD30L KO mice with experimentally-induced colitis such as colitis induced by transfer of
353 CD4⁺CD45RB^{hi} T cells in SCID mice and dextran sulphate sodium (DSS)-induced colitis in BALB/c
354 background mice [16, 25]. Soluble mCD30-Ig inhibited Th17 cell differentiation *in vitro*, and
355 ameliorated acute and chronic DSS-induced colitis in WT mice [25]. Thus, the modulation of
356 CD30L/CD30 signaling by soluble mCD30-Ig could be a novel biological therapy for inflammatory
357 diseases associated with Th17 responses. In the present study, mCD30-Ig treatment at the induction
358 phase ameliorated EAE by reducing the differentiation of MOG₃₅₋₅₅ peptide-specific Th17 cells in
359 dLNs. More importantly, even if mCD30-Ig was administered after clinical disease onset, inhibition of
360 the CD30L/CD30 axis resulted in milder paralysis at the chronic phase. The present study revealed

that modulation of CD30L/CD30 axis by soluble mCD30-Ig could be an attractive candidate for the treatment of MS.

TNF has a complicated role in the pathogenesis of EAE and MS. TNF neutralization by anti-TNF antibody ameliorated EAE [38, 39], however inhibition of TNF by mAb or TNF receptor p55 Immunoglobulin fusion protein exacerbated neurological symptoms of MS [40, 41]. Recently, it has become clear that two TNF receptors, TNFR1 and TNFR2 have opposite roles in EAE and selective inhibition of TNFR1 protected mice from EAE [42-44]. Given these complicated results, it is important to investigate carefully the occurrence of side effects during clinical trials of CD30L/CD30 axis modulation for MS.

5. Conclusion

In conclusion, the present study revealed the CD30L/CD30 axis was critically involved in pathogenic T cell responses not only at the induction phase in dLNs but also at the effector phase in the CNS. *In vivo* neutralization of CD30L by soluble mCD30-Ig fusion protein ameliorated clinical symptoms of EAE even when administered after disease onset. These results indicate that CD30L could be a new target molecule for the treatment of MS and other inflammatory CNS demyelinating diseases.

377 **Conflict of Interest**

378 Jun-ichi Kira is an advisory board member for Merck Serono and a consultant for Biogen Idec Japan. He has
379 received payment for lectures from Bayer Schering Pharma, Cosmic Cooperation, and Biogen Idec Japan. The
380 other authors have declared that no conflict of interest exists.

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389

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518 **Figure legends**

519 **Figure 1. CD30L KO mice are resistant to EAE.**

520 (A) Clinical scores in WT and CD30L KO mice are shown. The numbers in parentheses express
 521 disease incidence. Error bars represent mean $\pm$ standard error of the mean. (B) Histopathological
 522 analysis of spinal cords by hematoxylin and eosin staining was performed 18 days after immunization.
 523 Right panels show the magnified view of the area within the square in left panels. Magnification, left,
 524 $\times 40$; right, $\times 100$. (C) Klüver-Barrera's staining of spinal cord sections was performed on day 40.
 525 Arrowheads show demyelinated lesions. Magnification, $\times 40$. (D) The number of cells isolated from
 526 spinal cords 18 days after immunization was calculated by flow cytometric analysis. Absolute cell
 527 numbers of CD4+MHC Class II- (CD4 T cells, CD4), CD11b+MHC Class II+ (macrophage and
 528 microglia, Mac/Mic), and Gr-1+MHC Class II- (granulocytes, Gr) cells in the spinal cord are shown.
 529 Mean disease scores were 3.0 (WT, n=4) and 2.25 (CD30L KO, n=4). Error bars represent mean $\pm$
 530 standard deviation. Data are representative of three independent experiments. Statistical significance
 531 was evaluated by Student's *t*-test. *, $p < 0.05$, **, $p < 0.01$.

532

533 **Figure 2. CD30L/CD30 signaling contributes to antigen-specific proliferation and Th1 and Th17**
 534 **responses at the induction phase.**

535 The dLNs cells were harvested on day 9 after EAE induction and restimulated *in vitro* with either
536 medium alone or indicated concentrations of MOG₃₅₋₅₅ peptide. (A) Proliferation of dLNs cells in
537 response to MOG₃₅₋₅₅ peptide was examined by thymidine incorporation assay. (B) The percentage and
538 number of CD154⁺CD4 T cells in dLNs induced by restimulation of MOG₃₅₋₅₅ peptide. (C)
539 Intracellular cytokine staining of CD4 T cells after culture with MOG₃₅₋₅₅ peptide for 5 hours in the
540 presence of Brefeldin-A. (D) Concentrations of IL-17 and IFN- γ measured by ELISA in the culture
541 supernatant of dLNs cells (5×10^5) after restimulation by MOG₃₅₋₅₅ peptide for 3 days. (E) Cytokine
542 production of IL-17 and IFN- γ by CD4 T cells co-cultured with APCs from WT or CD30L KO mice.
543 Purified WT CD4 T cells (2×10^5) were cultured with 30 Gy-irradiated WT or CD30L KO splenocytes
544 in the medium with or without MOG₃₅₋₅₅ peptide for 72 hours. The concentrations of IL-17 and IFN- γ
545 were measured by ELISA. Data are representative of three independent experiments. Error bars
546 represent mean $\pm$ standard deviation. Statistical significance was evaluated by Student's *t*-test. * $p <$
547 0.05, ** $p < 0.01$.

548

549 **Figure 3. CD30L on hematopoietic cells and radioresistant cells is involved in the development of**
550 **EAE.**

(A) Three sets of BM chimera were prepared as follows: WT (Ly5.1/5.1) to WT (Ly5.2/5.2), WT (Ly5.1/5.1) to CD30L KO (Ly5.2/5.2), and CD30L KO (Ly5.2/5.2) to WT (Ly5.1/5.1). Recipient mice were lethally irradiated and transplanted with BM from donor mice. Active EAE was induced after confirming BM reconstitution 8 weeks later. (B) Clinical courses and (C) body weight changes are shown. The numbers in parentheses express disease incidence. Data are representative of two different experiments. Error bars represent mean $\pm$ standard error of the mean. Statistical differences were evaluated by Dunnett's test for multiple comparisons to control of WT $\rightarrow$ WT chimera. *, $p < 0.05$ (WT $\rightarrow$ CD30L KO chimera); †, $p < 0.05$; ††, $p < 0.01$ (CD30L KO $\rightarrow$ WT chimera).

Figure 4. CD30L KO mice are resistant to passive EAE.

The dLNs cells were harvested from WT mice 10 days after EAE induction and restimulated *in vitro* with MOG₃₅₋₅₅ peptide and recombinant mouse IL-23 for 3 days. Subsequently, cultured cells were intravenously transferred into 4 Gy-irradiated naive WT or CD30L KO mice (2×10^7 cells per recipient) followed by i.p. injection of PTX on day 0 and 2 after the transfer. (A) Clinical scores and (B) body weight changes are shown. The numbers in parentheses express disease incidence. (C) Histopathological analysis of spinal cords was performed on day 40 after the transfer by hematoxylin and eosin staining. Lower panels show a magnified view of the area within the square in upper panels.

Magnification, upper, $\times 40$; lower, $\times 100$. (D) Klüver-Barrera's staining was performed on day 40 after the transfer. Arrowheads show demyelinated lesions. Magnification, $\times 40$. Data are representative of three independent experiments. Error bars represent mean $\pm$ standard SEM. Statistical significance was evaluated by Student's *t*-test. *, $p < 0.05$.

Figure 5. Soluble mCD30-Ig treatment at the induction phase ameliorates actively induced EAE.

(A) Active EAE was induced in WT mice and they received 200 μ g of mCD30-Ig or PBS intraperitoneally on days 0, 2, 4, 6 and 8. (B) Clinical courses and (C) body weight changes are shown. The numbers in parentheses express disease incidence. Error bars represent mean $\pm$ standard error of the mean. (D) Histopathological analysis of spinal cords by hematoxylin and eosin staining were performed on day 17. Lower panels show the magnified view of the area within the square in upper panels. Magnification, upper, $\times 40$; lower, $\times 100$. (E) dLNs cells harvested on day 9 were restimulated *in vitro* with MOG₃₅₋₅₅ peptide for 5 hours in the presence of brefeldin-A. The expression of CD154 and (F) intracellular cytokine staining of CD4 T cells were examined by flow cytometry. Error bars represent mean $\pm$ standard deviation. Data are representative of three independent experiments. Statistical significance was evaluated by Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Figure 6. Soluble mCD30-Ig treatment after disease onset ameliorates EAE.

(A) Active EAE was induced in WT mice and they were administered 200 μ g of mCD30-Ig or PBS i.p. on days 12, 14, 16, 18 and 20. (B) Clinical courses and (C) body weight changes are shown. The numbers in parentheses express disease incidence. Error bars represent mean $\pm$ standard error of the mean. (D) Histopathological analysis of spinal cords was performed by hematoxylin and eosin staining on day 30. Lower panels show the magnified view of the area within the square in upper panels. Magnification, upper, $\times 40$; lower, $\times 100$. (E) Klüver-Barrera's staining was also performed on day 30. Arrowheads show demyelinated lesions. Magnification, $\times 40$. Data are representative of three independent experiments. Statistical significance was evaluated by Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Figure 1

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Figure 2

Figure 3

Figure 4

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Figure 5

Figure 6