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

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Abstract

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

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 γδ 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-κB) 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 MOG_{35-55} 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

MOG_{35-55} (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 MOG_{35-55} 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 MOG_{35-55} peptide emulsified with CFA. Ten days later, dLNs were harvested and single-cell suspensions were incubated in the presence of 10 μg/ml of MOG_{35-55} peptide and 10 ng/ml of recombinant IL-23 in a 37°C, 5% CO_2 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].

2.5. Thymidine incorporation assay

Whole dLNs cells were cultured in 96-well plates with 10 μg/ml of MOG_{35-55} peptide for 72 hours. Wells were pulsed with ^{3}H-thymidine (New England Nuclear, Boston, MA) at 37 mBq/well for the final 12 hours. Mean incorporation was measured by a 1205 Betaplate scintillation counter (PerkinElmer, Gaithersburg, MD).

2.6. Isolation of mononuclear cells from spinal cords

Spinal cords were excised from mice perfused transcardially with ice-cold phosphate buffered saline (PBS). They were cut into small pieces and digested with collagenase D (Invitrogen, Carlsbad, CA) and DNase I (DN25; Sigma-Aldrich, Tokyo, Japan). Dispersed cells were passed through a nylon mesh, placed onto 33% Percoll solution (GE Healthcare, Buckinghamshire, UK), and centrifuged at 800 ×*g* for 20 minutes. Cell pellets were suspended with culture medium and used for analysis.

2.7. Flow cytometric analysis and intracellular staining

For cell surface staining, single-cell suspensions were incubated with an optimal concentration of fluorescent Abs in Hanks' Balanced Salt Solution with 0.5% fetal bovine serum for 20 minutes at 4°C. Intracellular staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. In some experiments, we added propidium iodide (1 μg/ml) to cell 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 MOG_{35-55} 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 MOG_{35-55} 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 MOG_{35-55} 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 hematoxilin 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%

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

147 *2.11. Generation and in vivo administration of mCD30-Ig*

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

154 *2.12. Statistical analysis*

155 Statistical significance was evaluated by Student's *t*-test and Dunnett's test using JMP9 software (SAS,
156 Cary, NC). Differences with p values < 0.05 were considered statistically significant.
157

158 **3. Results**

159 *3.1. CD30L KO mice are resistant to active EAE.*

160 We examined the role of the CD30L/CD30 axis in the pathogenesis of active EAE induced by
161 immunization of mice with MOG_{35-55} peptide emulsified with CFA, followed by intraperitoneal (i.p.)
162 injection with PTX on days 0 and 2. WT mice started to develop EAE about 10 days after
163 immunization and reached a peak score at about 3 weeks with 100% incidence, whereas clinical
164 symptoms were significantly ameliorated in CD30L KO mice when compared with WT mice (Fig.
165 1A). Histological analysis of spinal cords was performed 18 days and 40 days after immunization for
166 EAE induction. Inflammatory cell infiltration was reduced in CD30L KO mice compared with WT
167 mice at the peak disease phase (Fig. 1B), and the severity of demyelination was attenuated in CD30L
168 KO mice at the chronic phase (Fig. 1C). To quantify the extent of cellular infiltration, flow cytometric
169 analysis was performed and revealed significantly impaired infiltration of $CD4^+$MHC Class II^- (CD4 T
170 cell), $CD11b^+$MHC Class II^+ (macrophage/microglia) and $Gr\text{-}1^+$MHC Class II^- (granulocyte) cells in
171 CD30L KO mice (Fig. 1D). Thus, the CD30L/CD30 axis is involved in the development of active
172 EAE.

173

174 *3.2. CD30L/ CD30 signaling is involved in MOG_{35-55} peptide-specific Th1 and Th17 responses at the*

induction phase in mice after immunization

To examine the role of the CD30L/CD30 axis in the development of MOG_{35-55} peptide-specific CD4 T cells, dLNs were harvested 9 days after immunization and were restimulated *in vitro* with or without various concentrations of MOG_{35-55} peptide. Ag-specific T cell proliferation was assessed by thymidine incorporation assay. Thymidine uptake was significantly reduced in CD30L KO mice compared with WT mice (Fig. 2A). Ag-specific T cells are reported to be defined by intracellular staining of CD154 after *in vitro* stimulation with Ag [30]. As shown in Fig. 2B, the frequency and number of $CD154^+$ CD4 T cells in dLNs was significantly lower in CD30L KO mice compared with WT mice. Furthermore, intracellular cytokine staining of a cell population gated on CD4+ T cells after restimulation with MOG_{35-55} peptide revealed that CD30L KO mice had significantly reduced levels of MOG_{35-55} peptide-specific Th1 and Th17 cells during the development of EAE (Fig. 2C). However, there were no differences in the percentages and cell numbers of Forkhead box p3 $(Foxp3)^+$ regulatory CD4 T cells in dLNs between WT and CD30L KO mice (Fig. S1). We further examined cytokine production from dLNs cells harvested on day 9 after immunization by enzyme-linked immunosorbent assay (ELISA) after 3 days culture in the presence of MOG_{35-55} peptide. Consistent with flow cytometric analysis, dLNs cells from CD30L KO mice produced significantly attenuated levels of IL-17 and interferon (IFN)-γ (Fig. 2D), whereas there were no differences in IL-2, IL-4, IL-10 and

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

We previously reported that $CD30^{+}$T-$CD30L^{+}$T cell interactions mediated Th1 cell responses in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) infection, and played a critical role in Th17 cell differentiation *in virto*, while CD30L expression on Ag-presenting cells (APCs) was dispensable in the development of Ag-specific Th1 and Th17 cells [16, 21]. To determine which cells are involved in mice developing EAE, we examined the expression pattern of CD30L. As previously described[8], CD30L was expressed on CD4 T cells, CD8 T cells, macrophages and DCs (Fig. S2). Unique $CD4^{+}CD3^{-}CD11c^{-}$ accessory cells were reported to be the constitutive source of CD30L [9]. However, we could not detect its expression. Subsequently, we cultured dLNs cells in a 96-well plate containing medium with MOG_{35-55} peptide (10 μg/ml), or in a plate coated by anti-CD3 mAb (5 μg/ml) for 24 hours, and examined CD30L expression. CD30L expression was enhanced by stimulation with MOG_{35-55} peptide, and almost all CD4 T cells expressed CD30L after stimulation by anti-CD3 mAb (Fig. S3). To address the possibility that CD30L on APCs is important for Ag presentation to CD4 T cells, we purified CD4 T cells from dLNs on day 9 after immunization and co-cultured them with 30 Gy-irradiated splenocytes from WT and CD30L KO mice as APCs in the presence of MOG_{35-55} peptide for 72 hours. There were no differences in IL-17 and IFN-γ production in the culture 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 $CCR6^{+}IL\text{-}17A^{+}$ 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 -> WT); (2) Recipient CD30L KO (Ly5.2/5.2) mice were lethally

irradiated and transferred with BM cells from WT (Ly5.1/5.1) mice (WT -> CD30L KO); and (3) Recipient WT (Ly5.1/5.1) mice were lethally irradiated and transferred with BM cells from CD30L KO (Ly5.2/5.2) mice (CD30L KO -> WT). After confirming BM reconstitution 8 weeks later, BM chimera mice were immunized with MOG_{35-55} peptide emulsified with CFA. As shown in Fig. 3B and C, development of EAE in CD30L KO->WT mice were significantly ameliorated compared with WT -> WT mice, confirming that CD30L expression on BM derived cells have a pivotal role in EAE development. Although the severity of EAE was comparable to WT -> WT mice, it was notable that the onset of EAE was significantly delayed in WT -> CD30L KO mice. These results suggest that CD30L expression on BM derived cells has a critical role but its expression on radioresistant host cells might at least partly be involved in EAE development.

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

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

(Ly5.1/5.2) were lethally irradiated and transferred with a mixture of BM cells from WT (Ly5.1/5.1) and CD30L KO (Ly5.2/5.2) mice. After confirming reconstitution 8 weeks after transfer, the chimera mice were immunized with MOG_{35-55} peptide emulsified with CFA. The mixed BM chimera mice developed mild EAE, probably due to the effect of artificial manipulation by bone marrow transplantation and the relatively old week-age recipient mice (Fig. S6B). On day 10 after immunization, dLNs cells were harvested and the expression of CD154, intracellular staining of IL-17A and IFN-γ in CD4 T cells were examined fter restimulation with MOG_{35-55} peptide for 5 hours. The frequencies of $CD154^+$CD4 T cells, IL-$17A^+$CD4 T cells and IFN-γ^+ CD4 T cells in CD30L-deficient CD4 T cells were comparable to those in WT CD4 T cells (Fig. S6C and D). Twenty days after immunization, we examined the chimerism of CD4 T cells from dLNs and spinal cords. The ratio of WT (Ly5.1/5.1) to CD30L KO (Ly5.2/5.2) CD4 T cells was similar between spinal cord-infiltrating cells and dLNs cells (Fig. S6E). These results indicate that reverse signaling via CD30L on CD4 T cells is dispensable for their differentiation into MOG_{35-55} peptide-specific CD4 T cells and infiltration into the spinal cord.

3.5. CD30L in the environment at the effector phase is involved in EAE development and accumulation 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 MOG_{35-55} 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 MOG_{35-55} 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 MOG_{35-55} peptide-specific CD4 T cells examined by CD154
279 staining and MOG_{35-55} 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 MOG_{35-55} 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, MOG_{35-55} 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 MOG_{35-55}

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

Similar to other members of TNFSF proteins [31], it was reported that CD30L might transmit reverse signals to downregulate IL-2 production in CD4 T cells [7, 15, 16]. Because IL-2 is reported to inhibit Th17 differentiation [32], CD30L reverse signaling on CD4 T cells might be also responsible at least in part for differentiation of Th17 cells via down-regulation of IL-2 production. However, IL-2 production by CD30L-deficient CD4 T cells in response to MOG_{35-55} peptide was not significantly increased in CD30L KO mice. Furthermore, mixed BM chimera experiments revealed that reverse signaling was dispensable at the induction phase in mice developing EAE.

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

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

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

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

We previously reported that both Th1 and Th17 responses in lamina propria of the intestine were impaired in CD30L KO mice with experimentally-induced colitis such as colitis induced by transfer of $CD4^{+}CD45RB^{hi}$ T cells in SCID mice and dextran sulphate sodium (DSS)-induced colitis in BALB/c background mice [16, 25]. Soluble mCD30-Ig inhibited Th17 cell differentiation *in vitro*, and ameliorated acute and chronic DSS-induced colitis in WT mice [25]. Thus, the modulation of CD30L/CD30 signaling by soluble mCD30-Ig could be a novel biological therapy for inflammatory diseases associated with Th17 responses. In the present study, mCD30-Ig treatment at the induction phase ameliorated EAE by reducing the differentiation of MOG_{35-55} peptide-specific Th17 cells in dLNs. More importantly, even if mCD30-Ig was administered after clinical disease onset, inhibition of 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.

Conflict of Interest

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

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References

[1] Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. Nature protocols, 2006;1:1810-9.

[2] Stromnes IM, Goverman JM. Passive induction of experimental allergic encephalomyelitis. Nature protocols, 2006;1:1952-60.

[3] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S *et al.* IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. Journal of immunology, 2006;177:566-73.

[4] O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC *et al.* Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. Journal of immunology, 2008;181:3750-4.

[5] Oyamada A, Ikebe H, Itsumi M, Saiwai H, Okada S, Shimoda K *et al.* Tyrosine kinase 2 plays critical roles in the pathogenic CD4 T cell responses for the development of experimental autoimmune encephalomyelitis. Journal of immunology, 2009;183:7539-46.

[6] Smith CA, Gruss HJ, Davis T, Anderson D, Farrah T, Baker E *et al.* CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. Cell, 1993;73:1349-60.

[7] Shimozato O, Takeda K, Yagita H, Okumura K. Expression of CD30 ligand (CD153) on murine activated T cells. Biochemical and biophysical research communications, 1999;256:519-26.

[8] Kennedy MK, Willis CR, Armitage RJ. Deciphering CD30 ligand biology and its role in humoral immunity. Immunology, 2006;118:143-52.

[9] Kim MY, Gaspal FM, Wiggett HE, McConnell FM, Gulbranson-Judge A, Raykundalia C *et al.* CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. Immunity, 2003;18:643-54.

[10] Sun X, Shibata K, Yamada H, Guo Y, Muta H, Podack ER *et al.* CD30L/CD30 is critical for maintenance of IL-17A-producing gammadelta T cells bearing Vgamma6 in mucosa-associated tissues in mice. Mucosal immunology, 2013;6:1191-201.

[11] Shanebeck KD, Maliszewski CR, Kennedy MK, Picha KS, Smith CA, Goodwin RG *et al.* Regulation of murine B cell growth and differentiation by CD30 ligand. European journal of immunology, 1995;25:2147-53.

[12] Gilfillan MC, Noel PJ, Podack ER, Reiner SL, Thompson CB. Expression of the costimulatory receptor CD30 is regulated by both CD28 and cytokines. Journal of immunology, 1998;160:2180-7.

[13] Lee SY, Lee SY, Kandala G, Liou ML, Liou HC, Choi Y. CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. Proceedings of the National Academy of Sciences of the United States of America, 1996;93:9699-703.

[14] Duckett CS, Gedrich RW, Gilfillan MC, Thompson CB. Induction of nuclear factor kappaB by the CD30 receptor is mediated by TRAF1 and TRAF2. Molecular and cellular biology, 1997;17:1535-42.

[15] Wiley SR, Goodwin RG, Smith CA. Reverse signaling via CD30 ligand. Journal of immunology, 1996;157:3635-9.

[16] Sun X, Yamada H, Shibata K, Muta H, Tani K, Podack ER *et al.* CD30 ligand/CD30 plays a critical role in Th17 differentiation in mice. Journal of immunology, 2010;185:2222-30.

[17] Romagnani S, Del Prete G, Maggi E, Chilosi M, Caligaris-Cappio F, Pizzolo G. CD30 and type 2 T helper (Th2) responses. Journal of leukocyte biology, 1995;57:726-30.

[18] Bengtsson A. The role of CD30 in atopic disease. Allergy, 2001;56:593-603.

[19] Polte T, Behrendt AK, Hansen G. Direct evidence for a critical role of CD30 in the development of allergic asthma. The Journal of allergy and clinical immunology, 2006;118:942-8.

[20] Sun X, Somada S, Shibata K, Muta H, Yamada H, Yoshihara H *et al.* A critical role of CD30 ligand/CD30 in controlling inflammatory bowel diseases in mice. Gastroenterology, 2008;134:447-58.

[21] Tang C, Yamada H, Shibata K, Muta H, Wajjwalku W, Podack ER *et al.* A novel role of CD30L/CD30 signaling by T-T cell interaction in Th1 response against mycobacterial infection. Journal of immunology, 2008;181:6316-27.

[22] Fuchiwaki T, Sun X, Fujimura K, Yamada H, Shibata K, Muta H *et al.* The central role of CD30L/CD30 interactions in allergic rhinitis pathogenesis in mice. European journal of immunology, 2011;41:2947-54.

[23] Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nature medicine, 2000;6:782-9.

[24] Dai Z, Li Q, Wang Y, Gao G, Diggs LS, Tellides G *et al.* CD4+CD25+ regulatory T cells suppress allograft rejection mediated by memory CD8+ T cells via a CD30-dependent mechanism. The Journal of clinical investigation, 2004;113:310-7.

[25] Sun X, Yamada H, Shibata K, Muta H, Tani K, Podack ER *et al.* CD30 ligand is a target for a novel biological therapy against colitis associated with Th17 responses. Journal of immunology, 2010;185:7671-80.

[26] Blazar BR, Levy RB, Mak TW, Panoskaltsis-Mortari A, Muta H, Jones M *et al.* CD30/CD30

ligand (CD153) interaction regulates CD4+ T cell-mediated graft-versus-host disease. Journal of immunology, 2004;173**:**2933-41.

[27] Nishimura H, Yajima T, Muta H, Podack ER, Tani K, Yoshikai Y. A novel role of CD30/CD30 ligand signaling in the generation of long-lived memory CD8+ T cells. Journal of immunology, 2005;175**:**4627-34.

[28] Veldhoen M, Hocking RJ, Flavell RA, Stockinger B. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nature immunology, 2006;7**:**1151-6.

[29] Bowen MA, Lee RK, Miragliotta G, Nam SY, Podack ER. Structure and expression of murine CD30 and its role in cytokine production. Journal of immunology, 1996;156**:**442-9.

[30] Frentsch M, Arbach O, Kirchhoff D, Moewes B, Worm M, Rothe M *et al.* Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nature medicine, 2005;11**:**1118-24.

[31] Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annual review of immunology, 2005;23**:**23-68.

[32] Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z *et al.* Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity, 2007;26**:**371-81.

[33] Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature reviews Immunology, 2003;3**:**569-81.

[34] Tompkins SM, Padilla J, Dal Canto MC, Ting JP, Van Kaer L, Miller SD. De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis. Journal of immunology, 2002;168**:**4173-83.

[35] Kivisakk P, Imitola J, Rasmussen S, Elyaman W, Zhu B, Ransohoff RM *et al.* Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. Annals of neurology, 2009;65**:**457-69.

[36] Kawakami N, Lassmann S, Li Z, Odoardi F, Ritter T, Ziemssen T *et al.* The activation status of neuroantigen-specific T cells in the target organ determines the clinical outcome of autoimmune encephalomyelitis. The Journal of experimental medicine, 2004;199**:**185-97.

[37] Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S *et al.* C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nature immunology, 2009;10**:**514-23.

[38] Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ *et al.* An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. The Journal of experimental medicine, 1990;172**:**1193-200.

[39] Baker D, Butler D, Scallon BJ, O'Neill JK, Turk JL, Feldmann M. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. European journal of immunology, 1994;24**:**2040-8.

[40] van Oosten BW, Barkhof F, Truyen L, Boringa JB, Bertelsmann FW, von Blomberg BM *et al.* Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. Neurology, 1996;47**:**1531-4.

[41] TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. Neurology, 1999;53**:**457-65.

[42] Nomura T, Abe Y, Kamada H, Shibata H, Kayamuro H, Inoue M *et al.* Therapeutic effect of PEGylated TNFR1-selective antagonistic mutant TNF in experimental autoimmune encephalomyelitis mice. Journal of controlled release : official journal of the Controlled Release Society, 2011;149**:**8-14.

[43] Brambilla R, Ashbaugh JJ, Magliozzi R, Dellarole A, Karmally S, Szymkowski DE *et al.* Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination. Brain : a journal of neurology, 2011;134**:**2736-54.

[44] Williams SK, Maier O, Fischer R, Fairless R, Hochmeister S, Stojic A *et al.* Antibody-mediated inhibition of TNFR1 attenuates disease in a mouse model of multiple sclerosis. PloS one, 2014;9**:**e90117.

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 ± 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 ×40; right, ×100. (C) Klüver-Barrera's staining of spinal cord sections was performed on day 40.
525 Arrowheads show demyelinated lesions. Magnification, ×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 ±
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.**

The dLNs cells were harvested on day 9 after EAE induction and restimulated *in vitro* with either medium alone or indicated concentrations of MOG_{35-55} peptide. (A) Proliferation of dLNs cells in response to MOG_{35-55} peptide was examined by thymidine incorporation assay. (B) The percentage and number of $CD154^{+}CD4$ T cells in dLNs induced by restimulation of MOG_{35-55} peptide. (C) Intracellular cytokine staining of CD4 T cells after culture with MOG_{35-55} peptide for 5 hours in the presence of Brefeldin-A. (D) Concentrations of IL-17 and IFN-γ measured by ELISA in the culture supernatant of dLNs cells (5×10^5) after restimulation by MOG_{35-55} peptide for 3 days. (E) Cytokine production of IL-17 and IFN-γ by CD4 T cells co-cultured with APCs from WT or CD30L KO mice. Purified WT CD4 T cells (2×10^5) were cultured with 30 Gy-irradiated WT or CD30L KO splenocytes in the medium with or without MOG_{35-55} peptide for 72 hours. The concentrations of IL-17 and IFN-γ were measured by ELISA. Data are representative of three independent experiments. Error bars represent mean ± standard deviation. Statistical significance was evaluated by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

Figure 3. CD30L on hematopoietic cells and radioresistant cells is involved in the development of 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 ± standard error of the mean. Statistical differences were evaluated by Dunnett's test for multiple comparisons to control of WT -> WT chimera. *, $p < 0.05$ (WT-> CD30L KO chimera); †, $p < 0.05$; ††, $p < 0.01$ (CD30L KO -> 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 MOG_{35-55} 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.

568 Magnification, upper, ×40; lower, ×100. (D) Klüver-Barrera's staining was performed on day 40 after
569 the transfer. Arrowheads show demyelinated lesions. Magnification, ×40. Data are representative of
570 three independent experiments. Error bars represent mean ± standard SEM. Statistical significance was
571 evaluated by Student's *t*-test. *, $p < 0.05$.
572
573 **Figure 5. Soluble mCD30-Ig treatment at the induction phase ameliorates actively induced EAE.**
574 (A) Active EAE was induced in WT mice and they received 200 μg of mCD30-Ig or PBS
575 intraperitoneally on days 0, 2, 4, 6 and 8. (B) Clinical courses and (C) body weight changes are shown.
576 The numbers in parentheses express disease incidence. Error bars represent mean ± standard error of
577 the mean. (D) Histopathological analysis of spinal cords by hematoxylin and eosin staining were
578 performed on day 17. Lower panels show the magnified view of the area within the square in upper
579 panels. Magnification, upper, ×40; lower, ×100. (E) dLNs cells harvested on day 9 were restimulated
580 *in vitro* with MOG_{35-55} peptide for 5 hours in the presence of brefeldin-A. The expression of CD154
581 and (F) intracellular cytokine staining of CD4 T cells were examined by flow cytometry. Error bars
582 represent mean ± standard deviation. Data are representative of three independent experiments.
583 Statistical significance was evaluated by Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.
584

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

586 (A) Active EAE was induced in WT mice and they were administered 200 μg of mCD30-Ig or PBS i.p.

587 on days 12, 14, 16, 18 and 20. (B) Clinical courses and (C) body weight changes are shown. The

588 numbers in parentheses express disease incidence. Error bars represent mean ± standard error of the

589 mean. (D) Histopathological analysis of spinal cords was performed by hematoxylin and eosin

590 staining on day 30. Lower panels show the magnified view of the area within the square in upper

591 panels. Magnification, upper, ×40; lower, ×100. (E) Klüver-Barrera's staining was also performed on

592 day 30. Arrowheads show demyelinated lesions. Magnification, ×40. Data are representative of three

593 independent experiments. Statistical significance was evaluated by Student's *t*-test. *, $p < 0.05$; **, $p <$

594 0.01.

595

596

Figure 1

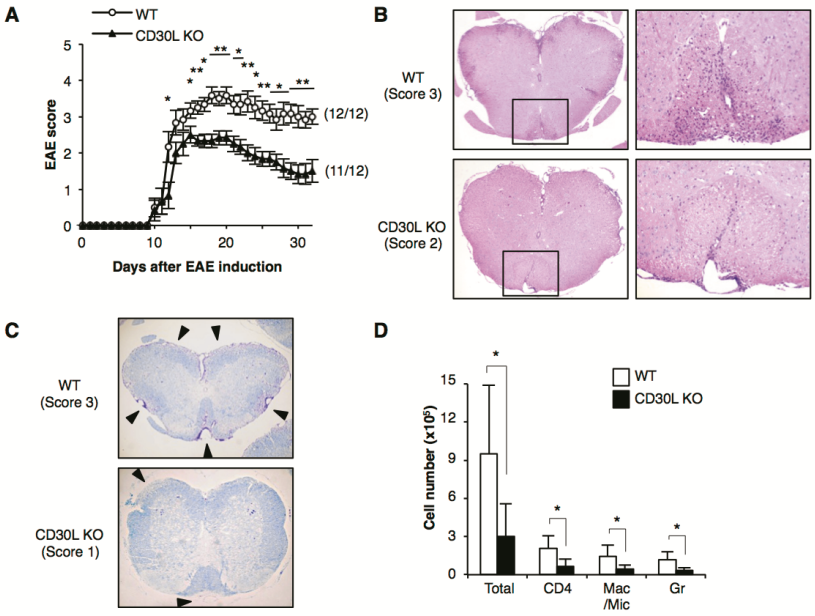

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Figure 2

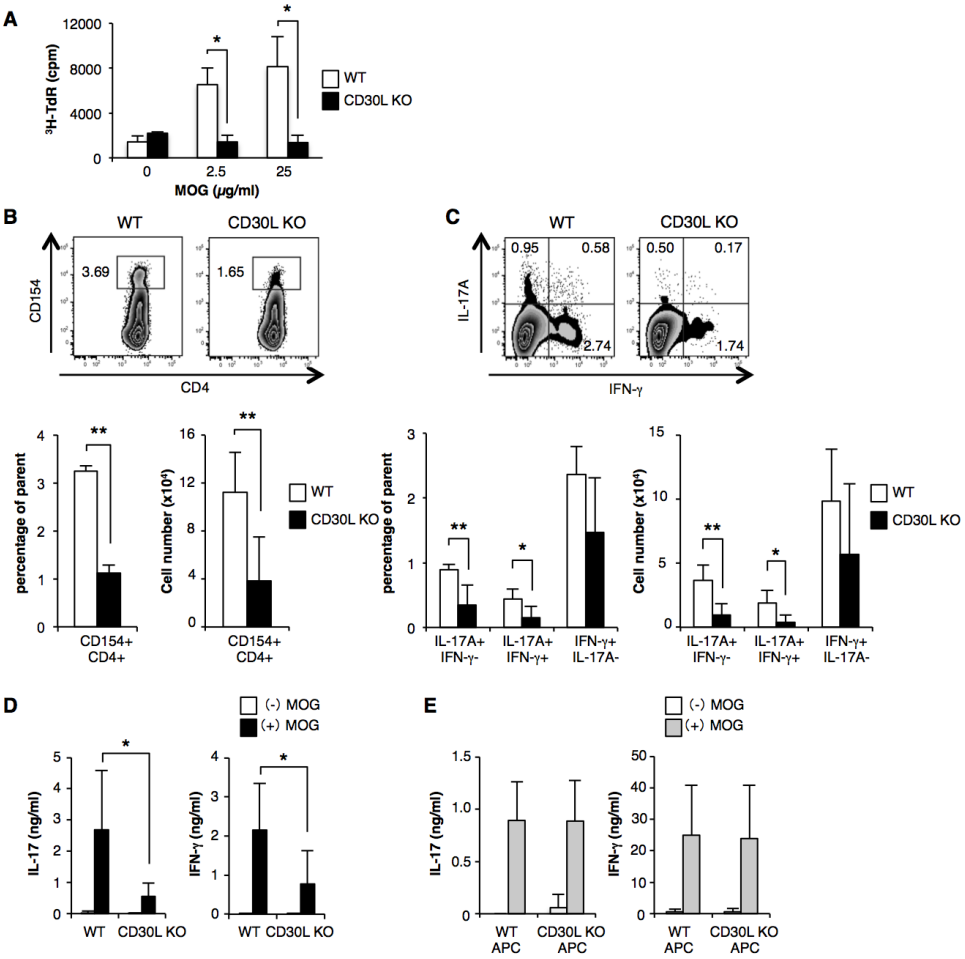

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Figure 3

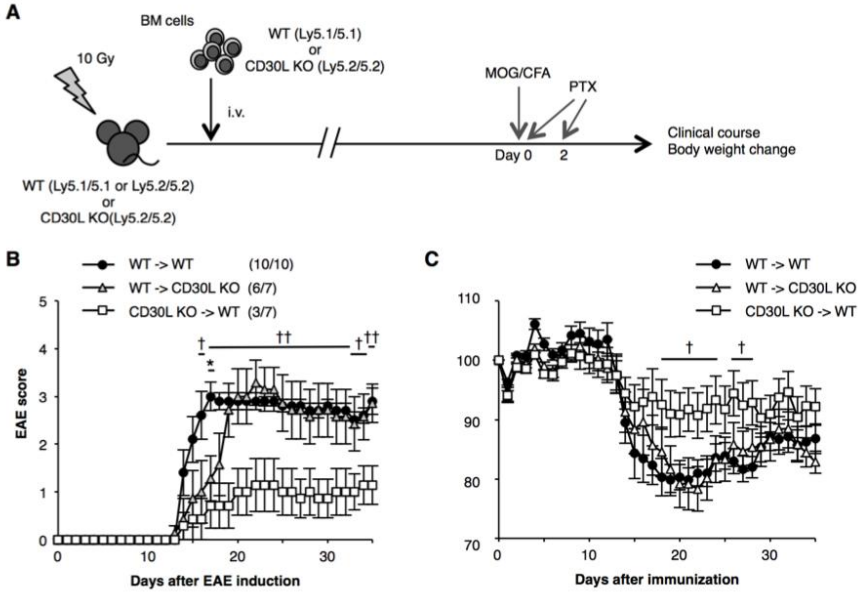

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Figure 4

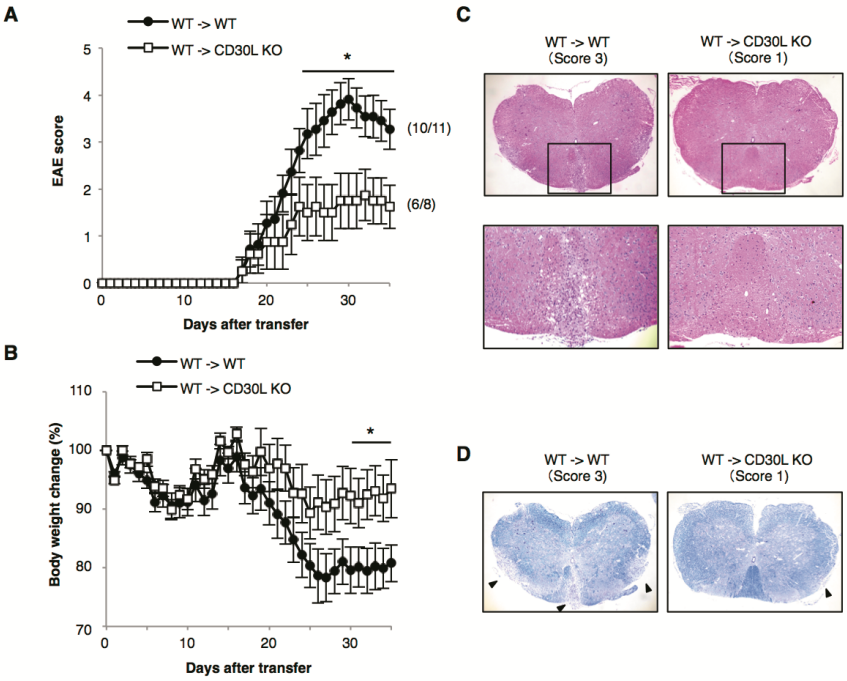

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Figure 5

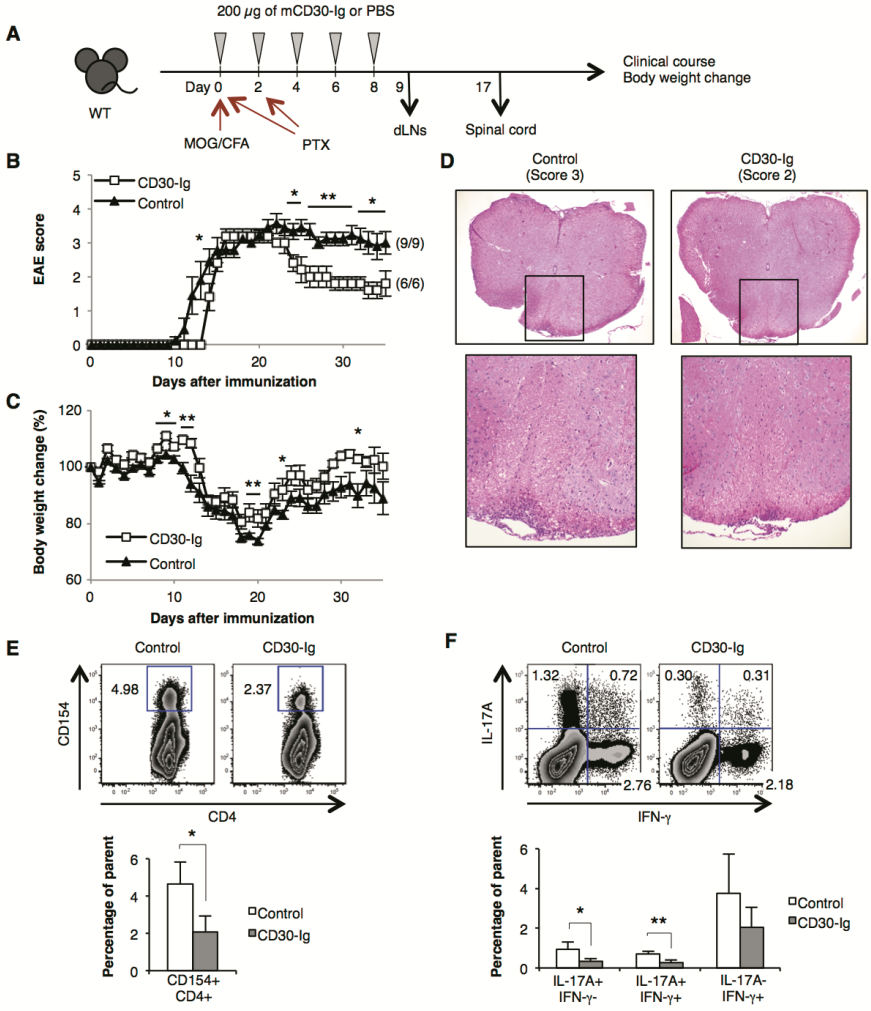

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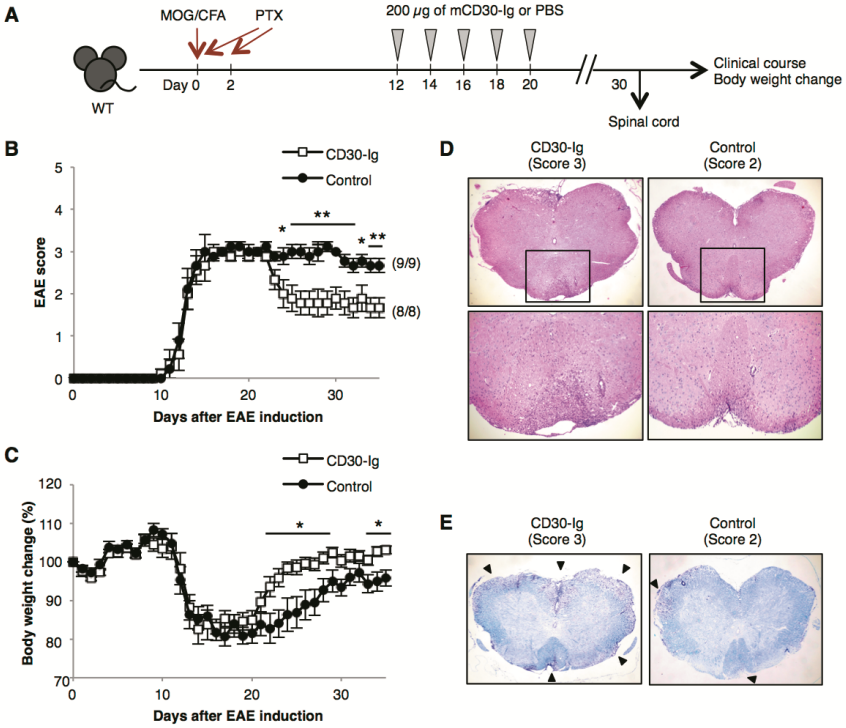
Figure 6


A
MOG/CFA
PTX
200 μg of mCD30-Ig or PBS
WT
Day 0
2
12
14
16
18
20
30
Spinal cord
Clinical course
Body weight change
B
CD30-Ig
Control
EAE score
Days after EAE induction
(9/9)
(8/8)
C
CD30-Ig
Control
Body weight change (%)
Days after EAE induction
D
CD30-Ig (Score 3)
Control (Score 2)
E
CD30-Ig (Score 3)
Control (Score 2)