博士論文

The Pathological role of DCIR in Autoimmune Encephalomyelitis in mice

(DCIR 欠損による中枢性自己免疫疾患の増悪化)

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I. Abstract

Dendritic cell immunoreceptor (DCIR) is a member of C-type lectin receptors which have a carbohydrate recognition domain on its extracellular domain and an immunoreceptor tyrosine–based inhibitory motif in its cytoplasmic portion, and which transduces negative signals to the cell. Previously, our group showed that DCIR-deficient (KO) mice spontaneously develop autoimmune diseases such as enthesitis and sialadenitis due to excess expansion of dendritic cells (DCs). This indicates that DCIR is critically important for the homeostasis of the immune system. In this report, I examined whether or not DCIR malfunction is involved in the development of an autoimmune disease, experimental autoimmune encephalomyelitis (EAE). EAE is an autoimmune disease model for human multiple sclerosis, in which not only encephalitogenic T cells but also other various immune cells are involved. I found that EAE was exacerbated in DCIR KO mice associated with severe demyelination of the spinal cord. In DCIR KO mice, the number of infiltrated $CD11c^+$ DCs and $CD4^+$ T cells in inflamed spinal cord was increased and $CD4^+$ T cell population in lymph node was expanded as compared to that in wild-type mice. Moreover, I found that DCIR expression is high in myeloid derived cells, especially in macrophages, in gene expression databases. Consistent with this, I found that inflammatory cytokine production was increased in bone-marrow derived macrophages from DCIR KO mice. These observations support the notion that DCIR is a negative regulator of the immune system to prevent excess activation.

II. Introduction

Autoimmune diseases are induced when the immune system improperly recognizes their self-molecules as antigens. Rheumatoid arthritis (RA) is one of typical autoimmune diseases and our laboratory has been investigating the target genes for the treatment of RA. In these processes, our laboratory had established two disease models of human RA; human T-cell leukemia virus type I (HTLV-I) transgenic (Tg) mice and interleukin-1 receptor antagonist (IL-1Ra) KO mice. Although the etiopathogenesises are different, both of these mice spontaneously develop autoimmune arthritis and the histopathology of the lesions closely resemble that of RA with marked synovial and periarticular inflammation caused by invasion of granulocytes[1,2]. To analyze the pathogenic mechanisms of RA, comprehensive analyses of gene expression between these two RA models and WT mice by microarray analysis revealed candidates for disease-related genes[3]. In that report from our laboratory, Fujikado *et al*. showed that the expression of 554 genes increased by three-fold in both RA model mice as compared to WT mice and some C-type lectin family genes were found as novel autoimmune disease-related genes. Among them, *Clec4a2* (also known as *Clecsf6*, and coded as *CLEC4A* or *CLECSF6* in human) which encodes DCIR was found. They showed that aged DCIR KO mice spontaneously develop sialadenitis and enthesitis, associated with elevated serum antibodies and DC expansion. Moreover, DCIR KO mice are more sensitive to collagen-induced arthritis (CIA), with expansion of DCs and antibodies against collagen and elevated responses of collagen-specific T cells[4]. DCIR inhibits the differentiation and proliferation of DCs by suppressing signal transduction and activator of transcription 5 (STAT5) phosphorylation[4] induced by granulocyte macrophage colony-stimulating factor (GM-CSF) signaling (Fig. 1). It is also known that human immunodeficiency virus type 1 (HIV-1) infects DCIR-expressing cells via the neck domain of DCIR[5,6]. Cross-linking of DCIR with antibodies inhibits TLR8-driven IL-12 and TNF-α production in

myeloid DCs and TLR9-driven IFN-α production in human plasmacytoid DCs[7,8] (Fig. 1). The suppressive effects of DCIR could be an indirect effect of ITIM on the Syk-coupled receptors which work synergistically with TLRs (Fig. 2). DCIR also drives antigen cross-presentation in human[9]. Moreover, polymorphisms of human DCIR (hDCIR) are associated with RA susceptibility and its expression was increased in inflamed joints[10-12]. These results indicate DCIR regulates various receptor signaling and cell functions.

Multiple sclerosis (MS) is one of neuroinflammatory diseases which are predominantly driven by $CD4^+$ T helper cells[13] (Fig. 3). These cells have multiple functions to regulate innate and adaptive immunity, activate other immune cells, and suppress immune reactions. MS development is thought to be mediated mainly by T cells. Naive T cells differentiate into their helper T cell subsets, such as type 1 T helper (Th1) cells, Th2 cells, and Th17 cells, by cytokines from antigen presenting cells (APCs) like DCs, and differentiated T cells exert their function in a subset specific manner. Th1 cells, which produce interferon (IFN)-γ, are differentiated from naive T cells with IL-12[14] and Th1 cells exist in the central nervous system (CNS) of EAE-induced mice[15,16]. Th1 was thought as the pivotal cells in CNS inflammation because treatment with an antibody to IL-12p40 could prevent the induction or progression of EAE[17]. However, IL-12p35 KO mice showed more severe symptoms than WT[18,19]. Moreover, IFN-γ KO mice or T cells could develop or induce EAE[20,21]. Therefore, these observations threw doubt on the notion that Th1 cell is responsible for EAE. IL-23 is an IL-12 family cytokine and is responsible for the differentiation of naive T cells to Th17 cells while IL-23 enhances IL-17 production by Th17 cells[22] and maintains expression of IL-17[23]. IL-23 consists of p19 and p40 subunits, sharing the p40 subunit with IL-12. Anti-p40 monoclonal antibodies inhibit both IL-23 and IL-12[24-26]. Chen *et al*. demonstrated that blocking IL-23 with anti-IL-23p19 monoclonal antibodies can inhibit EAE[27] and IL-23p19 KO mice do

not develop EAE[28-30]. Th17 cells infiltrate to the inflamed CNS of EAE-induced mice[31,32] and IL-17 or its receptor KO mice have been reported to show a reduced inflammation and severity of EAE[33,34]. IL-23-stimulated $CD4^+$ T cells can induce passive EAE[35], and blocking IFN-γ and IL-17 production with the antagonists for peroxisome proliferator-activated receptors inhibit the development of EAE[36]. Moreover, myelin antigen sensitized Th17 cells induce more severe EAE than Th1 cells following adoptive transfer to naive mice. In a variety of EAE models[29,37] and human MS are associated with increased IL-17 expression[38,39]. Therefore, Th17 cells are actually pathogenic cells in CNS autoimmune disease[29,40]. GM-CSF is also essential cytokine for the pathogenicity of effector T cells. GM-CSF upregulates expression of major histocompatibility complex class II (MHC II) molecules and cytokines in granulocytes and macrophages then activate T cells indirectly[41]. GM-CSF induces inflammatory cytokine production of T cells and GM-CSF KO T cells could not initiate CNS inflammation[42,43]. Actually, GM-CSF deficiency results in impaired T cell expansion, Th17 cell differentiation[44-46], and myeloid cell accumulation in the CNS[45,47]. Moreover, in the EAE model, administration of GM-CSF neutralizing mAb at the onset of disease is effective in blocking further development of disease symptoms[43]. Therefore, GM-CSF plays a critical role in the CNS inflammation.

TNF-α is also produced by Th17 cells, monocytes and macrophages and can induce inflammatory responses[48,49]. Soluble TNF receptor (TNFR) 1 protein or anti-TNF-α antibody can prevent the development of EAE as the onset of disease was delayed in TNF-α deficient mice. However, the incidence and severity of disease in these mice was comparable to that in WT mice, which suggests an important role of TNF-α for disease initiation[50-52]. Other studies reported that TNFR KO mice exhibit symptoms which were milder or absent in TNFR1 KO or TNFR1/2 double KO than WT mice, whereas TNFR2 KO mice showed more severe symptoms and demyelination and a higher inflammatory responses.

Moreover, TNFR2 mediated protective actions were demonstrated, such as myelin regeneration and lymphocyte suppression. It seems that TNFR1 plays an inflammatory role, while TNFR2 engages a suppressive role in CNS inflammation[50-52]. However, in contrast to TNF-α inhibition in mouse EAE, the opposite effects were observed in human MS[53,54]. DCs controls the activation and polarization of T cells and is known as a professional APC[55] (Fig. 3). In CNS autoimmunity, DCs secrete chemokines that promote T cell invasion and reactivation in the inflammatory loci[56]. Epitope spreading is thought to be important in CNS autoimmunity, which occurs from sequence similarities among antigens and self-peptides that are enough to result in the cross-activation of autoreactive T or B cells[57]. $F4/80^{-}$ $CD11c^{+}$ $CD45^{hi}$ DCs infiltrate the CNS and enhance epitope spreading in EAE[58]. It is also reported that CNS-infiltrating $CD11b^{+}$ DCs polarize naive T cells to Th17 cells[59,60]. On the contrary, plasmacytoid DCs (pDCs) limit the differentiation of effector Th1 and Th17 cells and do not affect the differentiation of $FoxP3^{+}$ regulatory T cells[61]. In MS patients, there is a large number of DCs that accumulate in the white matter and the cerebrospinal fluid[62]. In RRMS and secondary progressive MS (there is no remission phase and this occurs after relapsing-remitting periods), circulating DCs show an activated state and produce much higher levels of IL-12p70 and IL-23p19[63-65]. Moreover, in RRMS patients, circulating DCs show higher expression of activation markers and C-C chemokine receptor type 5 (CCR5) than healthy controls[66,67]. The expression of the CCR5 ligands Chemokine (C-C motif) ligand 3 (CCL3) andCCL5 is increased in MS inflammatory lesions[68]. After TLR9 activation, circulating pDCs from RRMS patients produce decreased amounts of IFN-α. Furthermore, pDCs from MS patients do not affect regulatory T cells[69]. Therefore, DCs play a part of commander and this is more critical than T cells in this neuro-inflammatory disorder.

B cells contribute to the both degradation and protection of myelin through the production of

autoantibodies and the opsonisation of myelin debris[70,71] (Fig. 3). In acute, chronic active/inactive lesions, 50-75% of MS patients have high levels of IgG and IgM. These immunoglobulins appear independent of disease duration or clinical stage. Double immunofluorescence staining revealed that IgG and IgM are present on axons and oligodendrocytes where demyelination was found with complement[72]. B cells primed by Th2 cells produce anti-inflammatory cytokines like IL-4 and IL-13[73,74]. B cells are also thought to have a regulatory role in EAE with B-cell deficient mice which are unable to recover[75]. Anti-CD20 administration one week before EAE induction augmented disease severity and increased the infiltration of autoreactive T cells into the CNS[76]. These symptoms resulted from the loss of IL-10 or IL-35 production, which are known to be important for recovery from EAE[77,78]. On the other hand, a pathogenic role of B cells in EAE is suggested. B cells from RRMS patients secrete toxic factors to oligodendrocytes[79]. Moreover, B cells primed by Th1 cells secrete proinflammatory cytokines such as IFN-γ, IL-12 and TNF-α[73,74]. With an anti-CD20 antibody injection prior to EAE induction daily for three days, B cells are depleted and disease onset is significantly suppressed[80]. This is associated with less severe demyelination and a decrease in the production of Th1 and Th17 cells specific for myelin oligodendrocyte glycoprotein (MOG) [76,81]. Moreover, the pathogenicity of B cells in EAE is linked to their production of IL-6. Barr, *et al.* have shown that B cells from mice with EAE and human with MS produce more IL-6 than controls and anti-IL-6 antibodies could suppress this pathogenicity[82]. These data indicate B cells have important roles in the disease onset and development. Monocyte-derived macrophages accumulate in the CNS of the both MS patients and EAE induced mice although MS is characterized as a T-cell mediated disease[83,84] (Fig. 3). Activation of macrophages is also important for the progression of EAE. Although inhibition of macrophage activation before the disease induction had only limited effects on disease progression, inhibition at the onset of disease significantly ameliorated disease

symptoms[85]. Moreover, blocking monocyte infiltration strongly suppressed the progression of EAE[83]. Targeting estrogen receptor β[86] and using microRNA-124[87] inactivated macrophages including CNS resident microglia, ameliorated disease severity and improved recovery. There are two subtypes of macrophages; the proinflammatory M1 cells and the anti-inflammatory M2 cells. The former one is $iNOS^+$ and secretes TNF-α and IL-1β, the latter one is $Arg1^+$ and secretes IL-10[88-90]. These subtypes of macrophages could be found during the course of EAE and the M1/M2 ratio correlates with the disease severity[91-93]. Furthermore, the suppression of M1 macrophage accumulation in the CNS could suppress disease severity and demyelination in EAE[94]. Adoptive transfer of M2 cells interrupt $CD4^+$ T cell activation and ameliorates disease severity in EAE induced mice[95]. Therefore different macrophage populations also regulate EAE disease course.

Pattern recognition receptors (PRRs) perform a basic role in the innate immune system. These receptors that are expressed by immune cells identify pathogen-associated molecular patterns (PAMPs) which are associated with microbial pathogens or cellular stress and damage-associated molecular patterns (DAMPs) which are associated with damaged cell components. Toll-like receptors (TLRs) and C-type lectin receptor (CLR) are two major classes of PRRs[96] (Fig. 2). TLRs were discovered in *Drosophila*[97] and initiate the synthesis and secretion of cytokines and activation of other immune responses which are necessary for host defense. TLRs interact with their specific PAMP and then induce NF-κB signaling and the MAP kinase pathway which leads to the secretion of pro-inflammatory cytokines and co-stimulatory molecules. These secreted molecules signal to other cells of immune system and, therefore, make TLRs key elements of immunity[98]. In mammals, there are eleven TLRs and the importance of TLR2, TLR3, TLR4, TLR7 and TLR9 in CNS inflammation has been discussed[99,100]. TLR2 is expressed as a homodimer or a heterodimer, partnered with TLR1 or TLR6 on monocytes,

macrophages and myeloid DCs. TLR2 can bind a wide range of ligands including lipoteichoic acid of Gram-positive bacteria, bacterial lipopeptides and glycolipids, fungal beta glucan (zymosan) and the endogenous DMAPs Hyaluronan, HSP70 and HMGB1. TLR2 is found on CNS endothelial cells, microglia, astrocytes and oligodendrocytes and on infiltrating cells in MS patients[101,102]. Moreover, TLR2 is upregulated on peripheral bone marrow cells, cerebrospinal fluid mononuclear cells and in demyelinating lesions of MS[101,103-106]. MS patients relapse during bacterial infections[107]. Monocyte-derived DCs from MS patients with bacterial infections express high levels of HLA-DR and costimulatory receptors and exhibit higher levels of IL-12, IL-17 and IFN-γ[108]. High numbers of macrophages and microglia expressing the endogenous TLR2 ligand are also found in acute and RRMS[109]. Because MS lesions contain hyaluronan, oligodendrocyte precursor cells (OPCs) which contain hyaluronidases inhibit the maturation of OPC and remyelination via TLR2 ligation[106,110,111]. The TLR2 ligand zymosan can regulate the severity of MS. Inducing peripheral blood DC from MS patients when treated with IFN-β to secrete IL-10 which subsequently suppresses IL-23 and IL-1β production.[112]. Similarly, TLR2 expression on B cells and DC was significantly higher in helminth-infected MS patients than uninfected patients. In contrast, immunization with *S. pneumonia* exacerbated MS[113]. TLR3 is expressed on DC and B cells. This receptor recognizes viral double-stranded RNA. TLR3 signaling activates NF-κB to increase production of type 1 IFNs. Various neuronal cells express TLR3 such as cerebral endothelial cells[102], neurons, microglia, astrocytes and oligodendrocytes[101,103,114,115]. Stathmin, which is an endogenous TLR3 ligand, was found in astrocytes, microglia, and neurons of MS patients' brain, and was shown on the same set of neuroprotective elements as the synthetic TLR3 agonist polyinosinic: polycytidylic acid by cDNA microarrays[116]. However, association studies could not identify any relationship between *TLR3* sequence variants and MS[117,118]. Monocytes and macrophages, myeloid

DC and T and B lymphocytes express TLR4 which recognize LPS from Gram-negative bacteria, bacterial and endogenous heat shock proteins, fibrinogen, heparan sulphate and hyaluronic acid. Moreover, *TLR4* expression is detected in cerebral endothelial cells[102] and microglia by RT-PCR[101]. TLR4 and its ligand high mobility group box 1 are both increased in expression in the CSF mononuclear cells of MS patients[109]. However, association studies of missense mutations in *TLR4* failed to identify any association with MS[119,120]. TLR7 binds to viral single-stranded RNA and is expressed in monocytes, macrophages, pDCs and B cells. *TLR7* expression has been identified in microglia by RT-PCR[101]. As mentioned above, IL-17 performs a critical role in the pathogenesis of MS and EAE[16,27,32] and type I IFNs regulate IL-17 production[121,122]. IFN-β1a can induce the expression of TLR7 on human monocyte-derived DCs *in vitro* and its downstream signaling (MyD88 etc.), but inhibits the *IL1R* and retinoic acid-related orphan nuclear hormone receptor C and *IL-17A* gene expression and IL-17A secretion[123]. TLR9 is expressed on monocytes, macrophages, pDC, and B cells and recognizes unmethylated CpG DNA. TLR9 expressing pDCs are found in the leptomeninges and demyelinated tissue of MS patients. They are a main source of type I IFN, and secrete IFN-α in response to TLR9 stimuli[124]. This response is upregulated in untreated MS patients[125] and IFN-β treatment reduces the expression of TLR9 in MS patients[126]. Moreover, IFN-β inhibits TLR9 activation and IFNα secretion by pDC in all MS patients[125,127].

Among TLRs mentioned above, TLR2, 4, and 9 KO mice were examined for their susceptibility to EAE[99]. EAE in *Tlr2* KO mice is operator dependent. While some groups reported *Tlr2* KO mice develop a normal clinical course of disease[128,129], other groups showed a mild decrease in female *Tlr2* KO mice[130,131]. Conflicting results have been published on the disease progression of EAE in TLR4 KO mice. TLR4 KO C57BL/6 mice that were immunized with recombinant rat MOG protein showed increased *Il6* and *Il23* mRNA expression by myeloid DC, an increased production of Th17 cells and

increased EAE disease scores[132]. However, in mice that were immunized with MOG_{35-55} peptide, EAE disease scores were unaffected by targeted deletion of *Tlr4*[133,134]. Whereas *Tlr9* KO mice showed a decreased score of EAE induced with MOG_{35-55}[128], EAE was exacerbated in the KO mice treated with recombinant rat MOG[132]. Moreover, the EAE scores were reduced in female mice when disease was induced with MOG_{35-55}/CFA plus pertussis toxin[133]. CLRs recognize polysaccharide structures of pathogens and are expressed mainly in DCs, macrophages, B cells, monocytes, and neutrophils[135].

DCs express various types of CLRs both in human and mouse[136]. One or several carbohydrate-recognition domains (CRDs) are at the extracellular portion of CLRs, which bind to sugar structures of glycosylated antigen in a calcium-dependent manner. The particular motifs like immunoreceptor tyrosine-based inhibitory motif (ITIM), immunoreceptor tyrosine-based activating motif (ITAM) or ITAM-like motifs at the intracellular part, and most of the CLRs subsequently internalize antigen through receptor-mediated endocytosis and transduce signals (Fig. 2). With these signaling motifs, CLRs not only activate DCs for initiating immune responses but also maintain homeostasis of the immune system[136,137]. ITAM or ITAM-like motif containing CLRs are shown as "self-sufficient PRRs" which utilize spleen tyrosine kinase (Syk) [137,138]. Two tyrosines located in the ITAM are phosphorylated which then creates a site for Syk binding. This causes Syk activation and phosphorylation of a number of substrates which initiate a series of signaling event[139]. Syk transduce signals to nuclear factor-kappa B (NF-κB) and other signaling cascades like mitogen-activated protein kinase and nuclear factor of activated T-cells. DC-associated C-type lectin (Dectin)-1 is a receptor with a Ca^{2+}-independent CRD and ITAM-like domain[140,141]. This receptor is mainly expressed on macrophages, DCs, neutrophils and a subset of T-cells. Bone marrow-derived macrophages stimulated with mycobacteria produce TNF-α and IL-6 in Dectin-1 dependent or independent manner[142]. Dectin-1 recognizes β-1,3-glucans of fungi,

bacteria and plants[143], and sticks to zymosan (yeast cell wall), and to fungal or bacterial pathogens[144,145]. Moreover, Dectin-1 can bind to an endogenous ligand in T cells[146]. Although the ITAM-like motif can recruit and activate Syk[140,141], its tyrosine is not located in the appropriate position[146]. Two Dectin-1 molecules are thought to come together with ligand and provide a docking site for Syk in *trans*. Dectin-2 contains an ITAM containing CLR, which has a typical Ca^{2+}-dependent CRD and binds to high-mannose structures including α-mannans present in *Candida albicans*[147,148]. Dectin-2 is classified into ITAM-coupled CLRs and is expressed generally in myeloid cells such as macrophages and monocytes and DCs[149,150]. Despite its name, Dectin-2 has only 20% homology to Dectin-1[151] and is a type II transmembrane CLR which has a typical Ca^{2+}-dependent CRD. Dectin-2 recognizes high-mannose structures, including α-mannans in *Candida albicans*[147,148]. This receptor recruits an ITAM-bearing Fc receptor γ (FcRγ) chain and the phosphorylation of its tyrosines leads to Syk recruitment[149,152,153].

Macrophage inducible C-type lectin (Mincle) also associates FcRγ chain and recruits Syk[154]. Mincle is expressed mainly in macrophages and, with its typical CRD, binds to α-mannose on fungus and trehalose-6,6'-dimycolate on *Mycobacterium tuberculosis* (MT). Mincle additionally recognizes the endogenous ribonucleoprotein SAP130 on dead cells. Mincle induces neutrophil infiltration to damaged tissue areas and may be involved in tissue repair[154]. In contrast to activating receptors, there is also regulative receptors that possess an ITIM motif (Fig. 2). These receptors have been proposed to regulate myeloid cell activation[135,137]. Dendritic cell immunoreceptor (DCIR) is such a regulating receptor. In mouse, there are four DCIR homologs (mDCIR, mDCIR2, mDCIR3, and mDCIR4). Although ITIM is contained in mDCIR and mDCIR2[155], their full function is still unknown. DCIR signals by antibodies or the hepatitis C virus (HCV) which inhibit TLR8-induced IL-12 and TNF-α production by myeloid DCs and TLR9-mediated induction of IFN-α by pDCs[7,8,156] (Fig. 1). There are some CLRs which do not have

these signaling motifs, such as mannose receptor (MR, CD206) and DC-specific intracellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN, CD209) (Fig. 2). The MR has eight linked CRDs and one cysteine-rich domain and is mainly expressed on alveolar macrophages[157]. Mycobacterial stimulation through MR results in production of IL-4 and IL-13 and inhibition of IL-12 production, which fails to activate oxidative responses[158]. DC-SIGN performs an important role in MT recognition. This molecule is expressed mainly on DCs and serves as both a PRR and adhesion receptor to function in DC migration and DC-T cell interaction[159,160]. DC-SIGN activates the serine/threonine kinase Raf1[161] and regulates TLR-mediated NF-κB activation. Therefore, PRRs are major the targets of immune regulation.

Although a number of studies emphasize the potential relevance of immune cells and PRRs for CNS immune surveillance or autoimmune reactions, the contribution of DCIR in the initiation and perpetuation of neuroinflammation and disease symptoms remains unknown. In our recent study, there was much higher Th17-related cytokines in lymph node cell culture supernatant, which was extracted from CIA induced DCIR KO mice, and DCIR regulates GM-CSF signaling[4]. Th17 cells and these cytokines are important in EAE disease progression. Moreover, it has been reported that one member of CLR, CLEC16A, is upregulated in the etiology and/or progression of this disease[162,163]. Therefore, the DCIR should take a part in this disease. To examine this possibility, I examined the effects of DCIR deficiency on the development of EAE using DCIR KO mice. I show that the development of EAE is exacerbated in DCIR KO mice. Consistent with the nervous symptoms, infiltration of immune cells including $CD4^+$ T cells and $CD11c^+$ DCs into the spinal cord is greatly increased in DCIR KO mice at the late phase of EAE, with remarkable demyelination of the nerves in the white matter. Moreover, there is an augmented tendency for cytokine production of DCIR KO macrophages in vitro. These results suggest that DCIR is involved in the neuronal auto inflammation, not only in the adaptive immune system but

also in the innate immune system.

III. Materials and methods

i. Experimental animals

DCIR KO mice were backcrossed to C57BL/6J (Japan SLC, Japan) over twelve generations[4]. Gender- and age- matched WT C57BL/6J mice were purchased from Japan SLC. All mice were kept in specific pathogen–free conditioned rooms at the Center for Experimental Medicine and Systems Biology, the Institute of Medical Science, the University of Tokyo, and Research Institute for Biomedical Sciences, Tokyo University of Science. All animal experiments were approved by the committees for animal experiments of both universities and conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

ii. Induction and evaluation of EAE

Progressive EAE was induced in WT and DCIR KO mice by subcutaneous immunization with 300 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in 100 μl of incomplete Freund's adjuvant (IFA) (Thermo Scientific, USA) and phosphate buffered saline (PBS) (1:1) supplemented with 500 μg MT H37RA (Difco Laboratories Inc., USA) on day 0 and 7. MOG_{35-55} peptide was synthesized with a solid-phase peptide synthesis and purified by high performance liquid chromatography by Prof. Ohmi (the Institute of Medical Science, the University of Tokyo, Japan). Clinical signs of EAE were monitored for up to 30 days after immunization. The clinical score was determined on a scale of 0-5: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis and 5, moribund state. The mean clinical score was calculated by averaging the score of all of the mice in each group, including animals that seemingly did not develop EAE. Cumulative disease score is the total value of clinical scores of each mouse with EAE after immunization.

iii. Histological Analysis

At the day 30 after immunization, mice were anesthetized with avertin (Sigma-Aldrich co., USA) and perfused with PBS to rinse blood off from the spinal cords through the intracardiac route using a peristaltic pump set (ATTO, Japan), followed by perfusion with 10% formalin neutral buffer solution, pH 7.4. Spinal cords, which were embedded in paraffin (Sakura Finetek, Japan) with Tissue-Tek Uni-Cassette (Sakura Finetek), were cross-sectioned at the lumbar level with 6 μm thickness, using a tissue microtome (BIOCUT microtome; Leica, Germany, or Sledge Microtome IVS-410; Sakura Finetek). For light microscopy, serial sections were stained with hematoxylin and eosin (HE) (Sigma-Aldrich, USA) or Luxor fast blue (LFB) (Nacalai Tesque, Inc., Japan, or MUTO pure chemicals Co., LTD., Japan). Images were taken with BZ-9000 (KEYENCE, Japan). The inflammatory conditions were evaluated using all assessable HE stained tissue sections of the lumbar spinal cord, followed by the instructions described below. We determined inflammation scores as follows: 0, no inflammation; 1, cellular infiltration only in the perivascular areas; 2, one or two cellular infiltration loci in the white matter; 3, more than two cellular infiltration region in the white matter; and 4, the whole white matter is inflamed[164,165]. We calculated demyelination as follows; pictures of the spinal cord were taken after LFB staining, and the demyelinated area was determined with ImageJ software (National Institutes of Health, USA), and the ratio of demyelinated area/total area was calculated.

iv. Measurement of anti-MOG_{35-55} antibody titers in serum

The titer of anti-MOG_{35-55} antibodies was detected as described[166,167], with the following modifications. Briefly, 96-well plates were coated with 3 μg MOG_{35-55} peptide in per well by incubating at 4 °C

overnight. After substantial washing with PBS, 30 μl of 100-fold diluted sera was incubated in duplicates for 1 hr at room temperature. After washing, alkaline phosphatase-labeled goat anti-mouse immunoglobulins (ZYMED, USA) were reacted for 1 hr at room temperature, followed by the addition of p-nitrophenyl phosphate substrate (Sigma-Aldrich co., USA). The titer of anti-MOG_{35-55} antibody was given as an absorbancy at 415 nm value.

v. Purification of infiltrated cells in the spinal cords

EAE-induced mice were anesthetized with Avertin (Sigma-Aldrich co., USA) and perfused with PBS. Then, the spinal column from the cervical to the lumbar was removed by gross dissection. The spinal cord was ejected with an 18-gauge needle attached syringe and incubated in 200 U/ml collagenase from *Clostridium histolyticum* (Sigma-Aldrich co., USA) in Hank's balanced salt solution for 30 min at 37 °C. The cell suspension of dissociated CNS tissue in 30 % Percoll (Sigma-Aldrich co., USA) was overlaid on a 70 % Percoll in a 15 ml polypropylene tube and centrifuged at 2200 rpm for 20 min at room temperature. Cells were collected from the interface between 30 and 70 % Percoll layers.

vi. Flow cytometric analysis

For flow cytometric analysis, the cell surface and intracellular staining were carried out according to the standard techniques. Whole CNS infiltrated cells or 2.0×10^5 lymphocytes were stained with 2 μg/ml monoclonal antibodies after Fcγ receptor blocking with 2.4G2 antibody. After cell surface staining, cells were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % saponin, and then intracellular staining was performed. Anti-mouse CD11c (N418 or HL3), CD4 (RM4-5 or GK1.5), CD8 (53-6.7), CD40 (3/2, 3), CD80 (16-10A1), CD86 (GL-1), MHC II (M5/114.15.2), B220 (R3C-6B2), CD3

(145-2C11), F4/80 (BM8), IFN-γ (XMG1.2) and IL-17A (TC11-18H10.1) were purchased from Biolegend (USA), and PE conjugated IL-17A (TC11-18H10) and biotin-conjugated anti-mouse OX40L (RM134L) was purchased from BD Pharmingen (USA). PE/Cy7-conjugated streptavidin was available from BD Bioscience, (USA). Stained cells were detected with a FACSCanto II Flow Cytometer (Becton, Dickinson and Company, USA) and analyzed with BD FACS Diva (Becton, Dickinson and Company, USA) and FlowJo software (Tree Star, USA).

vii. MOG_{35-55}-specific T cell proliferation

WT and KO mice were subcutaneously immunized with 100 μg of MOG_{35-55} peptide emulsified in 100 μl of IFA (Thermo Scientific) and PBS (1:1) supplemented with 250 μg MT H37RA (Difco Laboratories Inc.) on day 0. They were intraperitoneally administrated with 200 μg of pertussis toxin (List Biological Labs, Inc., USA) in 100 μl PBS on day 0 and 2. Brachial and axillary LN cells were harvested at day 7 after immunization and cultured at 3×10^5 cells/well in 96-well culture plates with or without MOG_{35-55} for 3 days. [^{3}H]TdR (0.25 μCi/ml) was incorporated for the last 6 hr and the radioactivity was measured with a MicroBeta (PerkinElmer, Inc., USA).

viii. Bioinformatics analysis and data management

The information of mRNA expression was obtained from BioGPS (http://biogps.org/; 96 samples with 3 probes (1422013_at, 1425406_at and 1425407_s_at) for *Clec4a2*, and 84 samples with 2 probes (219947_at and 221724_s_at) for *CLEC4A*), and Reference database for Expression Analysis database at Laboratory for System Biology and Medicine (LSBM), at RCAST, The University of Tokyo (LSBM; http://www.lsbm.org/database/; 112 samples with 2 probes (219947_at and 221724_s_at) for *CLEC4A*).

The Immunological Genome Project (ImmGen, http://www.immgen.org/) raw data (220 samples for *Clec4a2*) was obtained from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as GSE15907. For the analysis with the data, Expression Console (Affymetrix, Inc., USA) was used for data extraction.

ix. Induction of bone marrow derived macrophages

Bone marrow cells were collected from femurs and tibiae and cells were seeded from the whole bone marrow cells were seeded into a Ø100-mm untreated dish with RPMI 1640 medium supplemented with 10 % (vol/vol) fetal bovine serum (FBS) and 20 ng/ml recombinant mouse macrophage colony stimulating factor (M-CSF) (R&D Systems, USA). On day 3, medium was replaced with10 ml of 10 % FBS – RPMI 1640 medium. At day 7, cell culture supernatant was discarded and attached cells were collected after versenization were counted cells and used for the stimulation. This method provides more than 90% of $F4/80^+$ macrophages.

x. Measurement of cytokine production of bone marrow derived macrophages

5×10^5 of bone marrow derived macrophages were cultured with 10 μg/ml MT H37RA (Difco Laboratories Inc.) in 24 well plate. After 24 hrs, the supernatants were collected and the concentration of TNF-α and IL-6 were measured with ELISA MAX™ Kit (Biolegend).

xi. Statistical Analysis

Two-tailed Student's t-test were used for statistical evaluation of all results except for the EAE cumulative incidence and clinical score; they were evaluated by chi-square test and the Mann-Whitney U-test, respectively.

IV. Results

i. EAE is exacerbated in DCIR KO mice

1. DCIR KO mice develop severer EAE compared to wild-type mice

To examine the pathological role of DCIR in EAE, I immunized WT and DCIR KO mice with MOG_{35-55} peptide and monitored their clinical signs of EAE. As a result, a significant increase in the number of DCIR KO mice developed EAE than WT mice after day 20 through day 27 (Fig. 4a). The clinical scores of KO mice were almost three times higher than that of WT mice from day 17 to 30 (Fig. 4b). KO mice scored twice as high as that of WT mice with the cumulative and maximum scores (Fig. 4c, d). Moreover, they developed EAE about two days earlier than WT mice (Fig. 4e). All these disease indexes indicate that DCIR deficiency enhanced EAE severity.

To investigate the effect of DCIR deficiency at the inflammatory site, a series of HE-stained tissue sections of the spinal cords from EAE-developed mice were examined. These sections showed that there was more severe infiltration in spinal cord of EAE-induced DCIR KO mice than WT mice (Fig. 5a). LFB staining revealed the white matter of spinal cords that were severely impaired in DCIR KO mice, and there were a greater number of weaker stains in the LFB stained white matter (Fig. 5b). In summary, the inflammatory score and demyelination area were quantified and these indexes demonstrate that DCIR deficiency enhanced CNS inflammation (Fig. 5c, d). These data support the thesis that disease severity is enhanced by DCIR deficiency.

It is reported that autoreactive antibodies are observed in the sera of MS patients[168]. Therefore, I collected sera from EAE induced mice and measured the titer of MOG_{35-55}-specific antibodies. The IgM-type anti- MOG_{35-55} antibodies were not augmented in the serum of EAE induced mice (Fig. 6). Antibody levels of total IgG, IgG1, and IgG2a subtype were comparable between WT and DCIR KO mice,

while IgG2b antibody levels were not detected. These results indicate that DCIR deficiency does not regulate the disease severity via autoantibody production. Although there was no antibody difference, DCIR KO mice are highly susceptible to the development of EAE. This indicates that DCIR negatively regulates the development of EAE without modulating autoantibody production ability by B cells.

2. The infiltration of $CD11c^+$ cells and $CD4^+$ T cells into the CNS was increased in DCIR KO mice

Since T cells produce cytokines and cause tissue specific destruction at the inflammation site of autoimmune disease, I tried to detect infiltration of $CD4^+$ T cells and $DCIR^+$ $CD11c^+$ DCs in spinal cords. I found a significant difference in the number and the population of lymphocytes and myeloid cells between WT and DCIR KO mice at the stage of disease exacerbation but there were no differences in the number of $B220^+$ cells. The number of infiltrated $CD4^+$ T cells and $CD11c^+$ DCs in the CNS was significantly increased in DCIR KO mice at day 20, where the clinical score of DCIR KO mice was significantly higher than that of WT mice (Fig. 7). Not only in the CNS, $CD4^+$ cells and $CD11c^+$ cells in the draining LNs were also increased in DCIR KO mice (Fig. 8). Moreover, though $CD11c^+$ cells could be characterized with the expression of activation/maturation markers such as CD40, CD80, CD86, MHC class II, or OX40L, there was no difference between the DCs from spinal cords of WT and DCIR KO mice (Fig. 9) and these markers were also similar in LN cells (Fig. 10). Since T cells are primed by DCs at LNs and then migrate to inflammatory sites, one reason for the above results might be due to a peripheral increase in lymphocytes that could cause an exacerbation of EAE symptoms without a significant change in DCs. To evaluate this possibility, flow cytometry analysis was performed on draining LNs of immunized mice at day 7. The data shows that $CD4^+$ T cells were certainly increased (Fig. 11) though their subpopulation was not changed (Fig. 12). The population of Th17 cells and Th1 cells in

LNs, which are considered to be important for the development of EAE[169-171], were similar between WT and KO mice (Fig. 12b). As the $CD4^+$ T cell population was increased in the draining LNs of immunized mice, the proliferative response of lymphocytes to MOG_{35-55} peptide could be enhanced. My fellow Dr. Maruhashi showed that, when cultured with MOG_{35-55} peptide, DCIR deficient lymphocytes which collected from the draining LNs at 7 days after immunization with pertussis toxin were significantly enhanced in DCIR KO mice as compared to WT mice (Fig. 13, unpublished observation). When immunized with pertussis toxin, DC production of IL-6, TNF-α, IL-12, and interferon-inducible protein is induced[172]. This report suggests DCIR might regulate DC activation and support the proliferation results. However, I couldn't detect any difference among activation markers of DCs from immunized mice (Fig. 9, 10).

ii. Cytokine production of DCIR deficient bone marrow macrophages is increased

1. Expression profiles of the DCIR gene in mice and human

Although increased DCIR gene expression was demonstrated in our autoimmune disease model mice[3], no counter parts of clinical data have not been available in human. First of all I studied the expression of *Dcir* gene both in mouse and human cells using the databases open to the public domain Gene expression data composing the mouse *Clec4a2* and human *CLEC4A* expression profiles at ImmGen, BioGPS and LSBM. Mouse and human data sets had similar but different datasets in terms of the cells analyzed and the probes that were used. Therefore, to compare their DCIR expression data, I ordered their data and compared each expression data. These expression databases shows that *Clec4a2* is found in DCs, myeloid derived granulocytes and macrophages (Fig. 14). In human case, *CLEC4A* is expressed in monocytes and macrophages rather than DCs (Fig. 15). DCs, granulocytes, monocytes, and macrophages

are all derived from myeloid cells[173], and monocytes become macrophages when they infiltrate to inflamed tissues. Moreover, depletion of macrophages ameliorates EAE[174]. These data suggested that the DCIR deficiency might affect macrophages.

2. DCIR deficient macrophages showed a tendency for increased cytokine production

To detect F4/80$^+$ macrophages in an inflamed spinal cord, I induced EAE in mice and found that there was no significant change among WT and DCIR KO in macrophages in LNs, spleen, and spinal cord at day 28 (Fig. 16) and LNs at day 7 following immunization (Fig. 17). The number and population are too small to detect any effect of DCIR deficiency. To clarify the effect, highly purified macrophages was needed and I induced macrophages from bone marrow cells with M-CSF[175,176]. With this protocol, high F4/80$^+$ bone marrow derived macrophages were obtained (over 90% of cultured cells were F4/80$^+$). The production of inflammatory cytokine TNF-α was significantly increased in the macrophages derived from DCIR KO mice when stimulated with desiccated MT. Moreover, IL-6 levels were found to be increased (Fig. 18). These pro-inflammatory cytokines activate and induce the migration of other leukocytes into the CNS and damage CNS tissue[177]. Therefore, DCIR deficiency also modifies cytokine production in macrophages.

V. Discussion

In this report, I showed that the development of EAE was significantly exacerbated in DCIR KO mice. The EAE scores assessed by several parameters were significantly increased by two-fold in KO mice (Fig. 4c, d), indicating that DCIR is an important regulator of EAE disease progression. I did not show DCIR expression in the inflammatory sites of EAE. However, as inflammatory cells including macrophages and DCs, which express DCIR, are accumulated in the spinal cord of diseased mice, DCIR expression should be upregulated in the inflamed sites. Though it is still not elucidated completely that the relationship between DCIR and the CNS inflammation both in mice and humans, EAE exacerbation in DCIR KO mice demonstrates the potential importance of DCIR in CNS inflammation in humans. Though I found that $CD11c^+$, $CD4^+$, $B220^+$ and $F4/80^+$ cells were accumulated in the CNS using flow cytometry, a question as to how these cells infiltrate these tissues still remains to be elucidated. Immunostaining with these antigens would address this and it will give us more information about the pathological processes in EAE.

Because autologous antibodies play an important role in the development of autoimmune diseases such as rheumatoid arthritis[178-181] and MS[76,168,182,183], I examined MOG_{35-55}-specific Ig concentration in EAE-induced mice. However, we found that serum levels of MOG_{35-55}-specific antibodies of the IgM, IgG1, IgG2a, and IgG2b subclasses were similar in DCIR KO mice and WT mice (Fig. 6). Consistent with these results, our laboratory previously reported that serum levels of MOG_{35-55}-specific Ig are not correlated with the development of EAE[33]. Furthermore, it was also reported that autoantibody levels are not always proportional to EAE disease scores[184]. These results suggest that antibody-mediated humoral immune responses are dispensable for the development of EAE in DCIR KO mice. Although the antibody production and cytokine production in CNS infiltrated B cells was not

examined, these cells could also be involved in the pathogenesis of EAE.

On the other hand, we found that the number of DCs and $CD4^+$ T cells infiltrated into the inflamed spinal cord was significantly increased in DCIR KO mice, supporting the notion that $CD4^+$ T cell-mediated cellular immune responses are important for the development of EAE. Pathogenic Th17 cells secrete GM-CSF then promote accumulation of inflammatory macrophages into the CNS and activate DCs to induce IL-23 production. The secreted IL-23 in turn promotes Th17 cell differentiation, forming a positive feedback loop. Moreover, our group has previously shown that DCIR negatively regulates DC differentiation and proliferation by suppressing GM-CSF receptor signaling through the ITIM in this molecule[4]. DCs are critical in the regulation of immune system[96]. They regulate appropriate reactions to various antigens by interacting with other immune cells especially T cells. Furthermore, DC regulation of undesirable responses is expected to have an advantage over standard treatments which can induce fatal side-effects[185,186]. Considering the major role of DCs in the induction and activation of effector T cells, DCs could be used to both suppress and induce immune responses in an antigen-specific manner. I found that the infiltrated $CD11c^+$ DCs were significantly increased in the inflamed CNS and LNs of DCIR KO mice (Fig. 7, 8). However, the expression of CD40, OX40L, CD80, CD86 and MHC class II molecules in DCs from DCIR KO mice was comparable to that of WT mice (Fig 9, 10), suggesting that DCIR does not regulate the expression of these receptors. Although we could not detect a preferential expansion of the Th17 subpopulation in LNs (Fig. 12), total $CD4^+$ cells in the spinal cord and LNs were significantly increased in these DCIR KO mice. Accordingly, total Th17 cell number in the spinal cord and draining LNs were increased in these mice and should promote inflammation after immunization with MOG peptides (Fig. 7). Thus, increased DC numbers which is caused by the hyper-responsiveness of DCs to GM-CSF would cause excessive antigen presentation and this could be

responsible for the augmented lymphocyte response against MOG_{35-55} immunization, as suggested in a previous report[4]. However, it has also been reported that some DCs located in lymphoid organs have the capacity to confer antigen-specific tolerance to T cells and protect from the development of MS[69,187,188]. By contrast, overproduction of cytokines by DCs can enhance autoimmune CNS inflammation[63,65,189]. Regarding DCIR mediated signal regulation, a previous report demonstrated that DCIR regulates TLR 8/9 signals in human pDC[7,8]. DCIR also affects macrophages which have TLR and CLR receptors like DCs (Fig. 18). Therefore, it will be interesting to examine whether or not the proliferation and cytokine production of mouse $CD11c^+$ DCs are controlled by MT stimuli.

Macrophages are also important in the first line of the immune system. Macrophages recognize and phagocytose pathogens to kill, and activated macrophages also produce cytokines to enhance inflammation. DCIR is expressed on macrophages[190], and, as I described, stimulated DCIR KO macrophages produce TNF-α and IL-6 more efficiently than WT cells (Fig. 18). In EAE, these cytokines are important for the development and progression of few disease[191,192]; TNF-α induces expression of chemokines[193,194] and IL-6 activates and enhances T cell differentiation and expression of adhesion molecules[192,195], although anti-TNF-α treatment exacerbates human MS[196]. Therefore, enhancement of this cytokine production up-regulates immune cell infiltration into CNS and could be another reason for the EAE augmentation in DCIR KO mice. However, the effect should be carefully considered in human cases. Moreover, macrophages also express various types of PRRs including mannose receptor, DC-SIGN, TLR2/4 and 9 and Dectin-1/2[197,198], and previous reports have demonstrated that DCIR regulates the expression of some of these receptors on DCs[7,8]. To confirm the regulatory roles of DCIR in macrophages, I examined the effects of TLR8/9 ligands on DCIR KO mouse-derived bone marrow macrophages. MT can stimulate various PRRs[197,198]. Especially, TLR9 should be regulated by DCIR in mouse macrophages

as found in human pDCs[8]. Therefore, TLR signaling under the control of DCIR is required to be assessed further. Also the expression of activation markers in different subtypes of macrophages should be explored. I found in this study M-CSF-induced bone marrow macrophages have potential to produce TNF-α while they exhibit the phenotypes of M2 macrophages which secrete anti-inflammatory cytokine IL-10[177] healing injured spinal cord[199]. Accordingly, the protective manner of M-CSF-induced bone marrow macrophages should be assessed in the future.

Collectively, these observations indicate that DCIR is crucial not only for the development of autoimmune arthritis but also for the development of encephalomyelitis. This suggests that DCIR regulation is highly important for the development of autoimmune diseases. Thus, the ligands or agonistic antibodies against DCIR would be a good candidate to treat autoimmune diseases.

VI. Figures

Figure 1

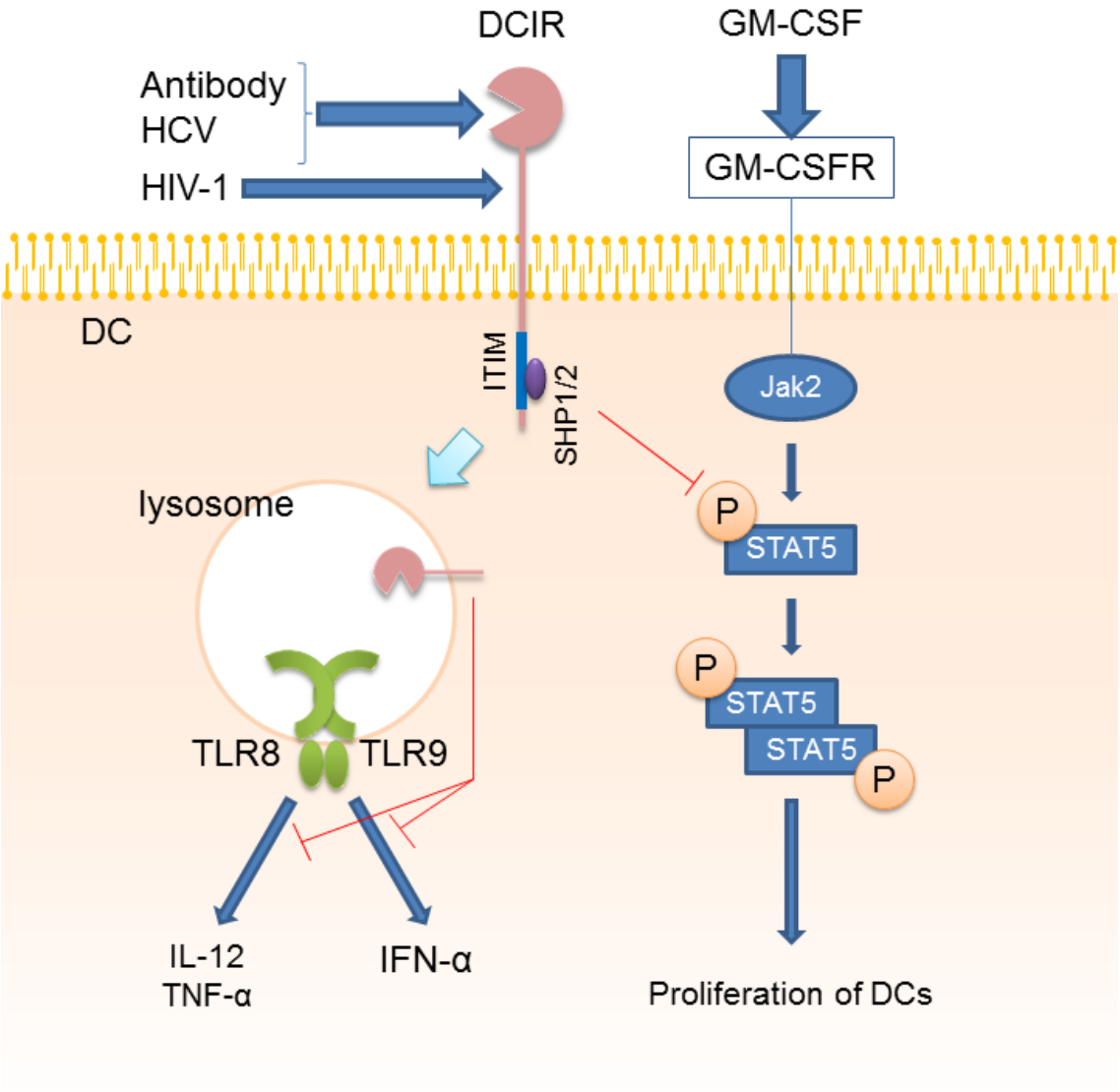

Figure 1 DCIR regulates TLR8/9 and GM-CSFR signals.

DCIR has a CRD and ITIM. Anti DCIR antibodies or HCV can stimulate DCIR. Though stimulated DCIR is internalized to the lysosome and inhibit cytokine production occurred with TLR 8 and TLR9 stimulation in human cells, the exact molecular which DCIR regulates is still unknown. DCIR also inhibits the differentiation and proliferation of DCs by suppressing GM-CSF signaling through blocking STAT5 phosphorylation. Moreover, HIV-1 binds to a neck domain of DCIR to infect into DCIR expressing cells. GM-CSFR: GM-CSF receptor.

Figure 2

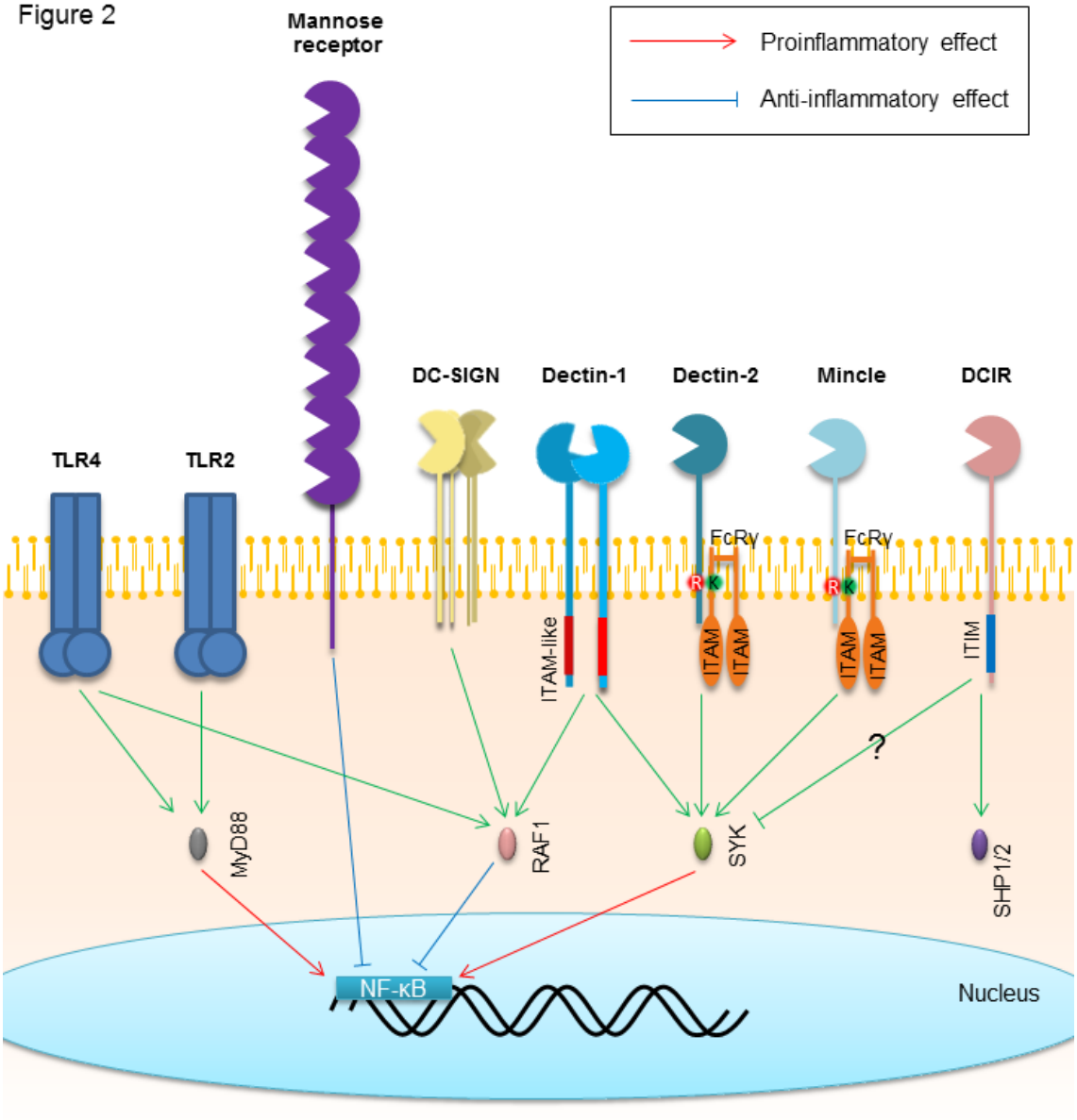

Figure 2 Pattern recognition receptors and their simplified signaling

These pattern recognition receptors (PRRs) recognize pathogens and induce intracellular signaling to the activation of transcription of NF-κB. After transcription, pro- and anti-inflammatory cytokines or chemokines are produced.

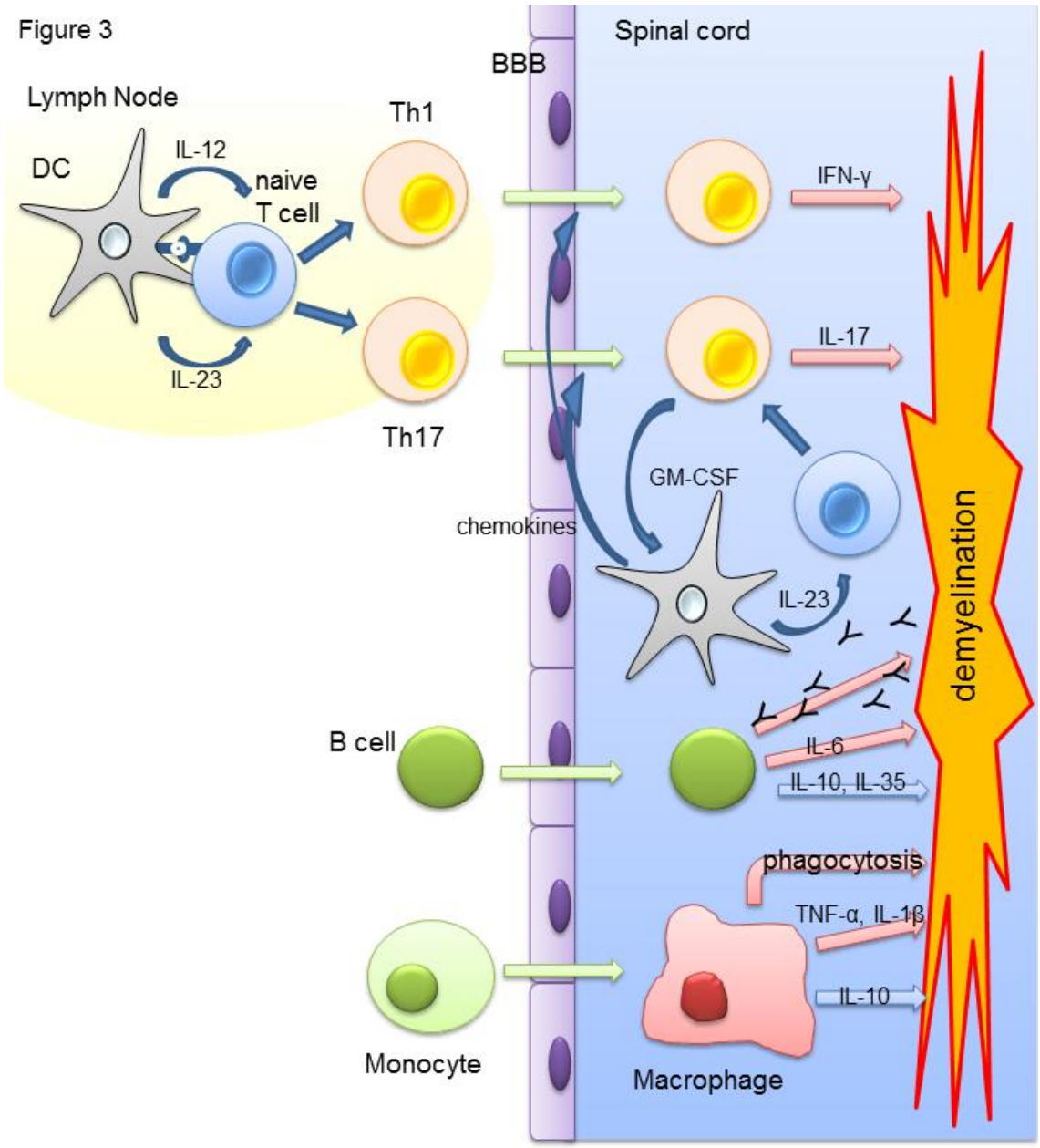

Figure 3 Scheme for immune cell contributions to the pathogenesis of EAE and MS

MS and its mice model EAE are Th17 cell dependent disease. T cells are differentiated by DCs followed by cytokine stimulation and antigen presentation which infiltrate in to CNS then destroy neuron tissue with production of inflammatory cytokines. Tissue resident DCs also promote T cell infiltration by chemokine production and naive T cell differentiation to Th17 cells at inflammatory site. B cells infiltrate in inflamed tissue and they produce immunoglobulins and cytokines to enhance or protect tissues. Circulating monocytes become macrophages when they infiltrate to CNS and phagocytose nerve tissue or produce cytokines to degrade neuronal tissue. Red and blue arrows represent enhance or suppress demyelination respectively. BBB: blood-brain barrier.

Figure 4

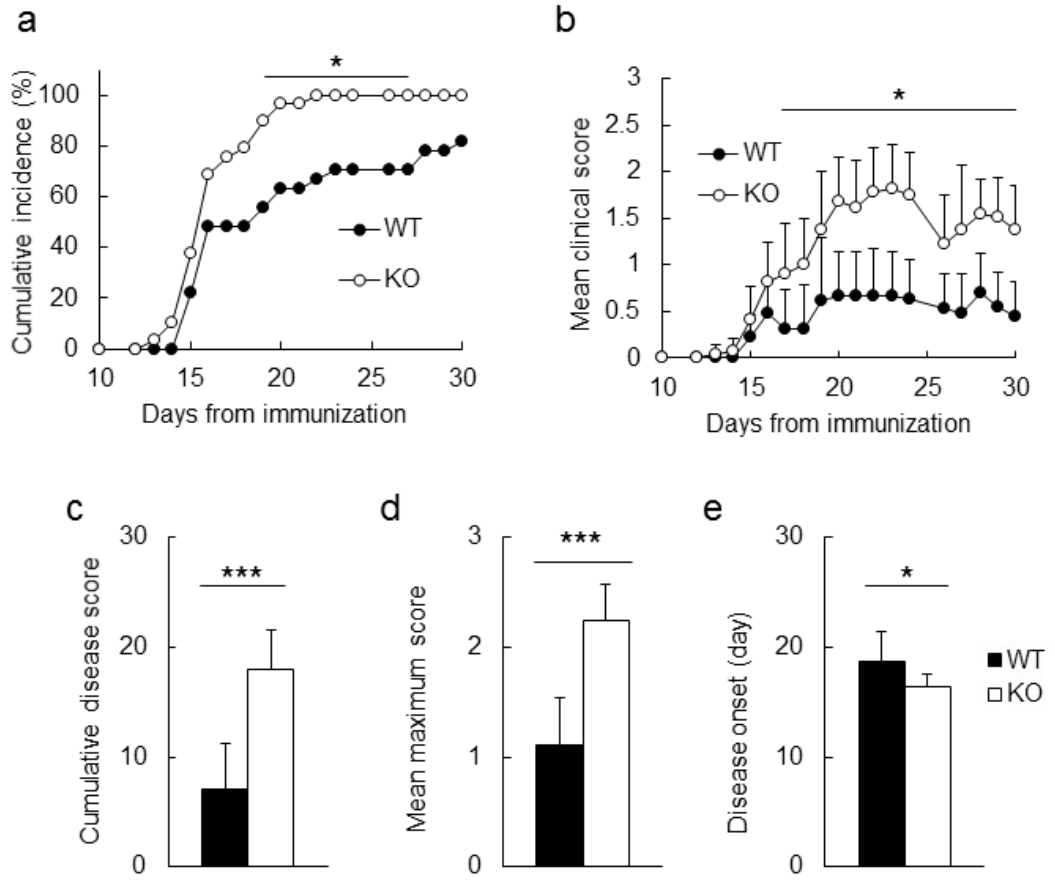

Figure 4 The EAE score is exacerbated in DCIR KO mice.

WT mice and DCIR KO mice were immunized and the development of neurological symptoms was monitored for 30 days. (a, b) The results show a combined data of 3 independent experiments with 5-16 mice for each. The total number of mice observed was 27 for WT mice and 29 for DCIR KO mice. The data is expressed as the means + standard error of the mean for each group. Statistical significance was determined using (a) chi-square test and (b) Mann-Whitney U test. (c) Cumulative disease scores in WT and DCIR KO mice. (d) Mean maximum score in each mouse over the course of the experiment. (e) The average days of disease onset in each group of mouse (*$P < 0.05$ and ***$P < 0.005$ vs WT mice score).

Figure 5

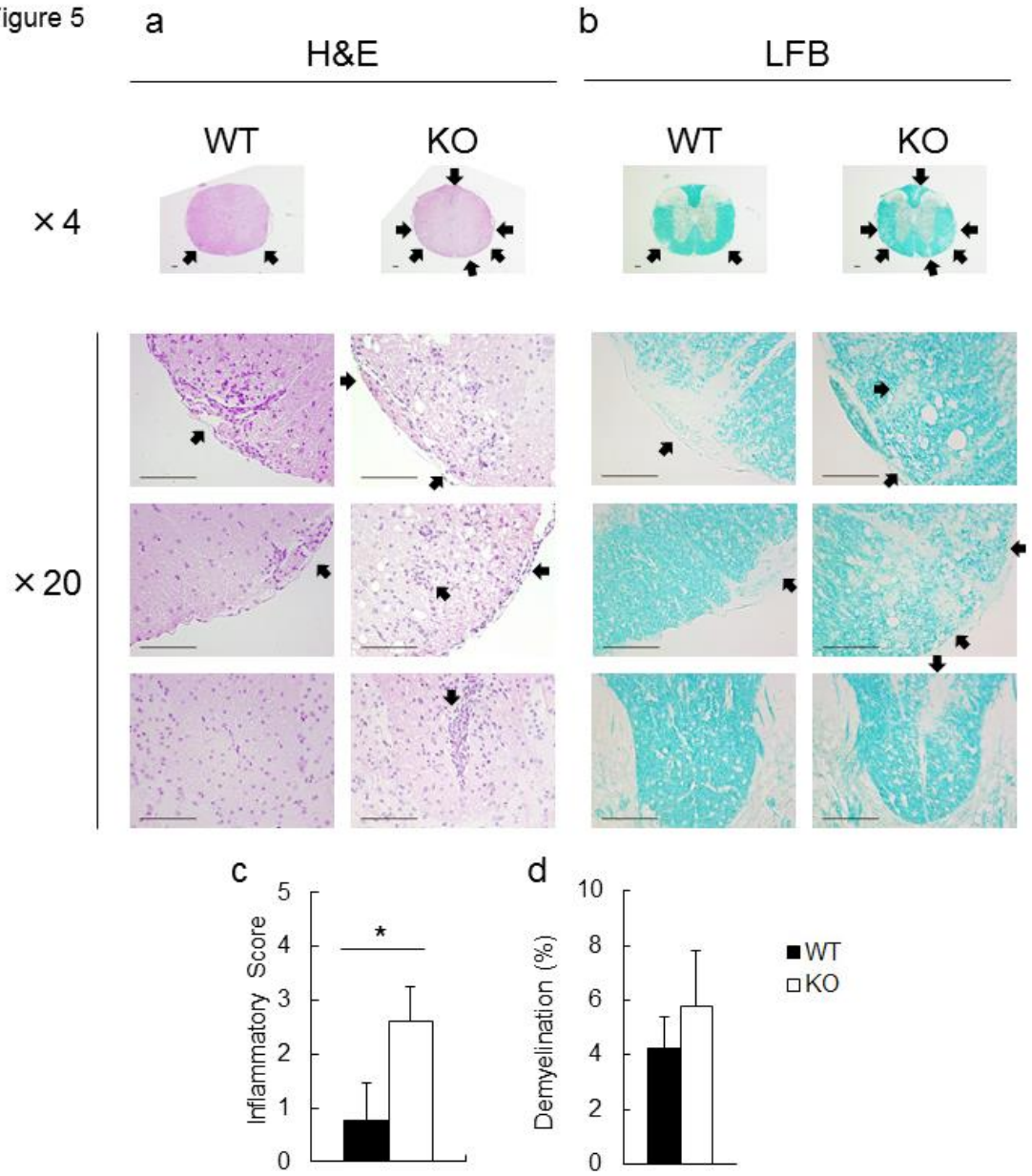

Figure 5 Histopathology of the spinal cords of EAE developed mice.

(a, b) At day 30 after immunization, tissue sections of the lumbar spine were stained with (a) HE or (b) LFB and arrowheads indicate (a) infiltration or (b) demyelination. The high-power field figures show the white matter near from the left and right ventrolateral sulcus and the center of posterior median septum. (c) Inflammatory score was scored and (d) demyelination ratio was calculated as described in materials and methods. Quantitative results were analyzed for 5 mice in each group, which are representatives of 2 experiments with similar results. Among the analysis, the same or serial sections were used and each graph represents average value and error bars represent the mean + standard deviation (SD).

Figure 6

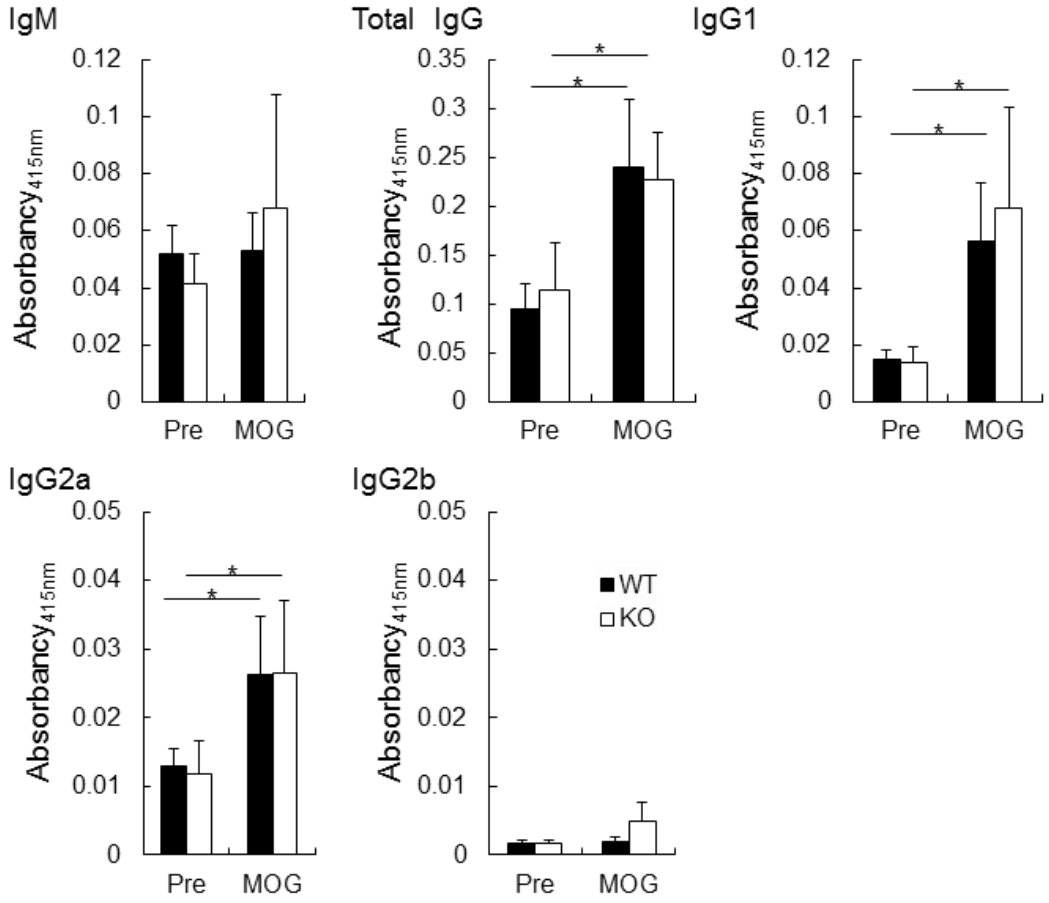

Figure 6 Antibody titers in the serum of EAE induced mice.

The sera were removed before immunization (Pre) and at 20 days after the first MOG_{35-55}/CFA immunization (MOG) (n=8), and the concentrations of each MOG_{35-55}-specific antibody were determined by ELISA. The data are expressed as means + SD, and are representative of two independent experiments (*$P < 0.05$).

Figure 7

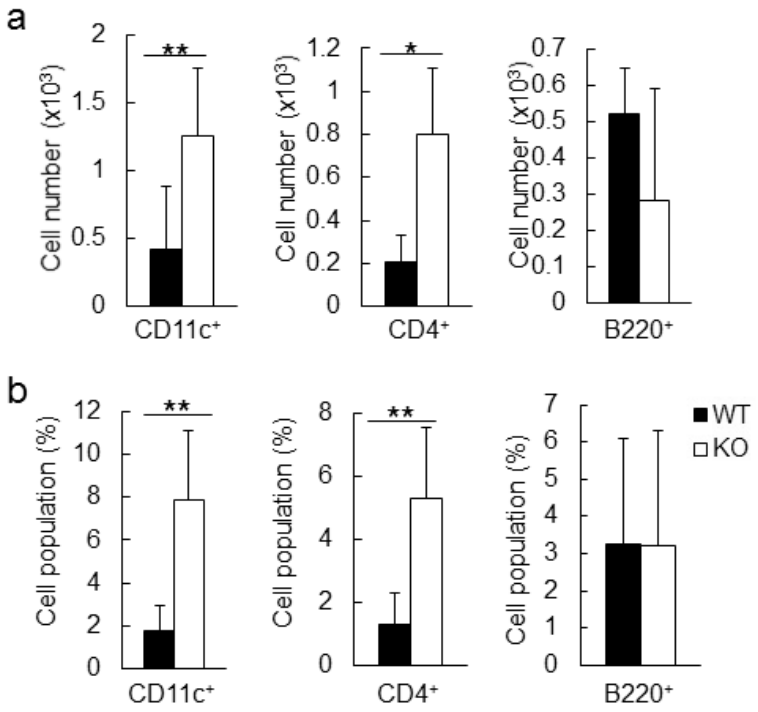

Figure 7 The infiltrated inflammatory cells are increased in DCIR KO mice.

(a) The numbers and (b) the population of infiltrated cells in the whole spinal cord were counted at 20 days after immunization (a; WT, KO: n =6 each, b; WT, KO: n= 9 each). The spinal cords were homogenized and cells were stained with antibodies against indicated antigens and analyzed by flow cytometry (*P < 0.05 and **P < 0.01 vs WT mice). The data are expressed as means + SD, and are combined results of two independent experiments.

Figure 8

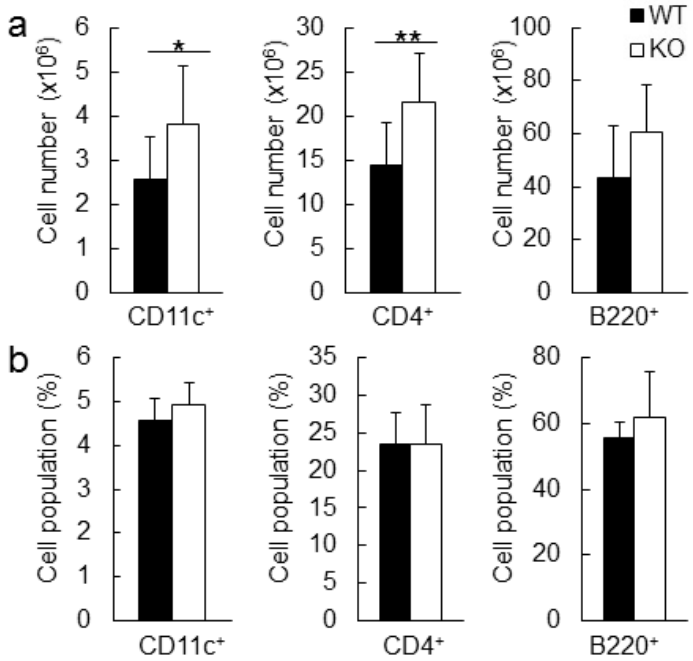

Figure 8 The number of immune cells is increased in DCIR KO mice.

(a) The numbers and (b) the population of immune cells in the LNs were counted at 20 days after immunization (n = 5). The draining LNs were homogenized and cells were stained with antibodies against indicated antigens and analyzed by flow cytometry. (*P < 0.05 and **P < 0.01 vs WT mice). The data are expressed as means + SD, and are representative of two independent experiments.

Figure 9

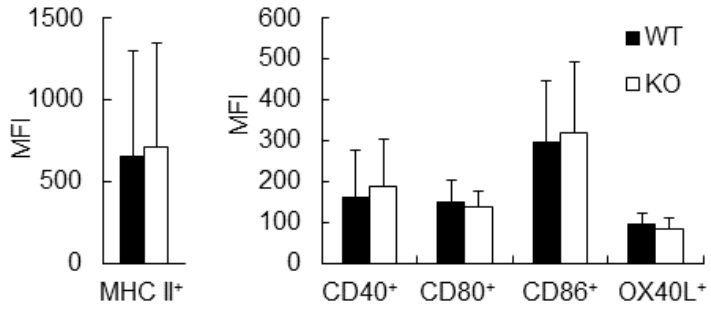

Figure 9 The activation markers of infiltrated DCs are similar in WT and DCIR KO mice.
The infiltrated cells in the whole spinal cord were collected at 20 days after immunization (n = 5). The spinal cords were homogenized and cells were stained with antibodies against indicated antigens and analyzed $7AAD^{-}$ $CD11c^{+}$ population by flow cytometry. The data are expressed as means + SD, and are representative of two independent experiments.

Figure 10

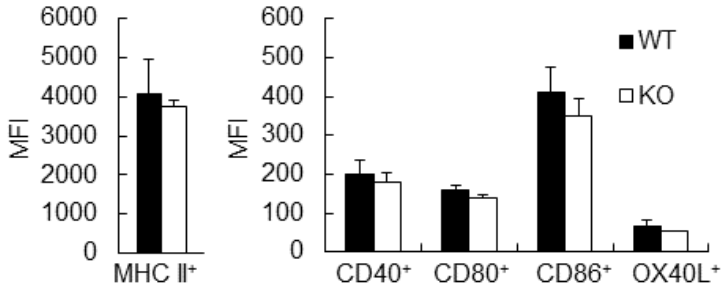

Figure 10 The activation markers of WT DCs are comparable to that of DCIR KO DCs.
The immune cells in LNs were collected at 20 days after immunization (n = 5). The LNs were homogenized and cells were stained with antibodies against indicated antigens and analyzed $7AAD^-$ $CD11c^+$ population by flow cytometry. The data are expressed as means + SD, and are representative of two independent experiments.

Figure 11

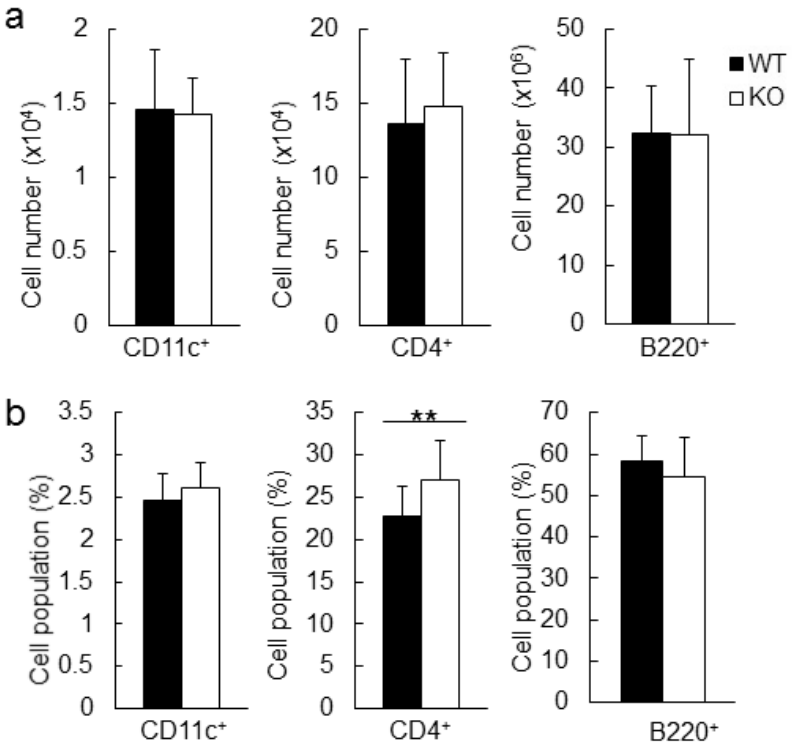

Figure 11 The population of $CD4^+$ T cells is expanded in draining LNs of DCIR KO mice after immunization.

Single cell suspensions were prepared from the draining LNs at 7 days after immunization. Cells were stained with antibodies against indicated antigens and analyzed by flow cytometry. The cell numbers and population of $CD11c^+$, $CD4^+$ and $B220^+$ cells were shown in (a) and (b) ($CD11c^+$ and $CD4^+$ cells are WT, KO; n = 15 each, and $B220^+$ cells are WT, KO; n = 11 each). The data are expressed as means + SD, and are combined results of three independent experiments.

Figure 12

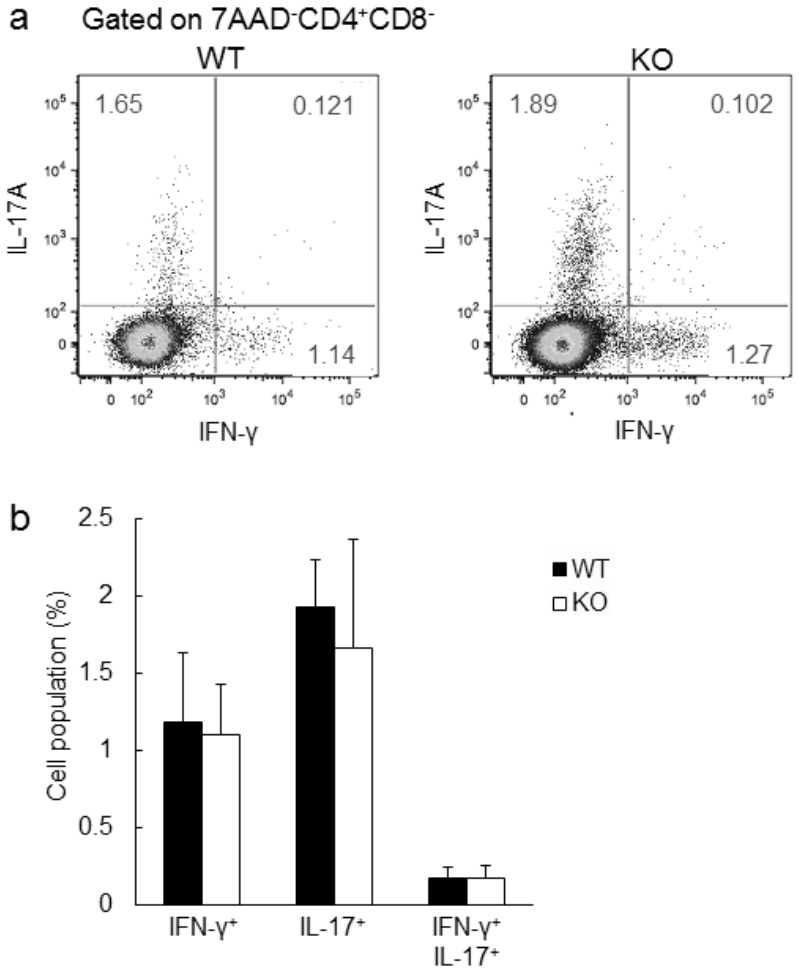

Figure 12 Cytokine expression in LN $CD4^+$ T cells is similar between WT and DCIR KO mice.
(A, B) Single cell suspensions were prepared from LNs at day 20 after MOG_{35-55} and CFA immunization, and after PMA/ionomycin stimulation, cells were stained with antibodies against indicated antigens and analyzed by flow cytometry (A). The population of IFN-γ^+, IL-17^+ and IFN-γ^+ IL-17^+ $CD4^+$ cells are shown in (B) (WT, KO: n = 8 each). The data are expressed as means + SD, and are combined results of two independent experiments.

Figure 13

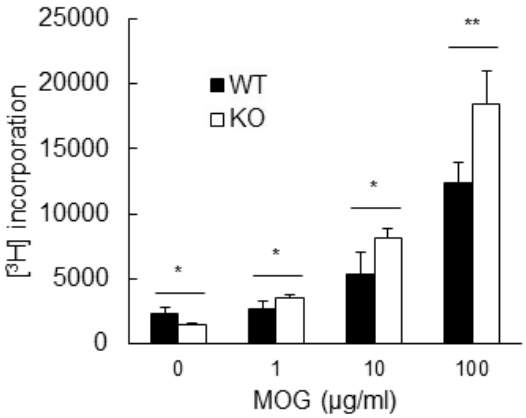

Figure 13 T cell recall proliferative response against MOG_{35-55} peptide is enhanced in lymphocytes from DCIR KO mice.

LN cells were harvested from mice (n = 6) at day 7 after immunization with MOG_{35-55}, CFA, and pertussis toxin. Pooled cells were incubated in the presence/absence of MOG_{35-55} peptide for 3 days. [^{3}H]TdR was incorporated over the last 6 hr, and the incorporation of [^{3}H] into acid insoluble fraction was measured. Data are presented as means + SD, and are representative of two independent experiments. *P < 0.05 and **P < 0.01 vs WT mice. This experiment was performed by Dr. Maruhashi.

Figure 14

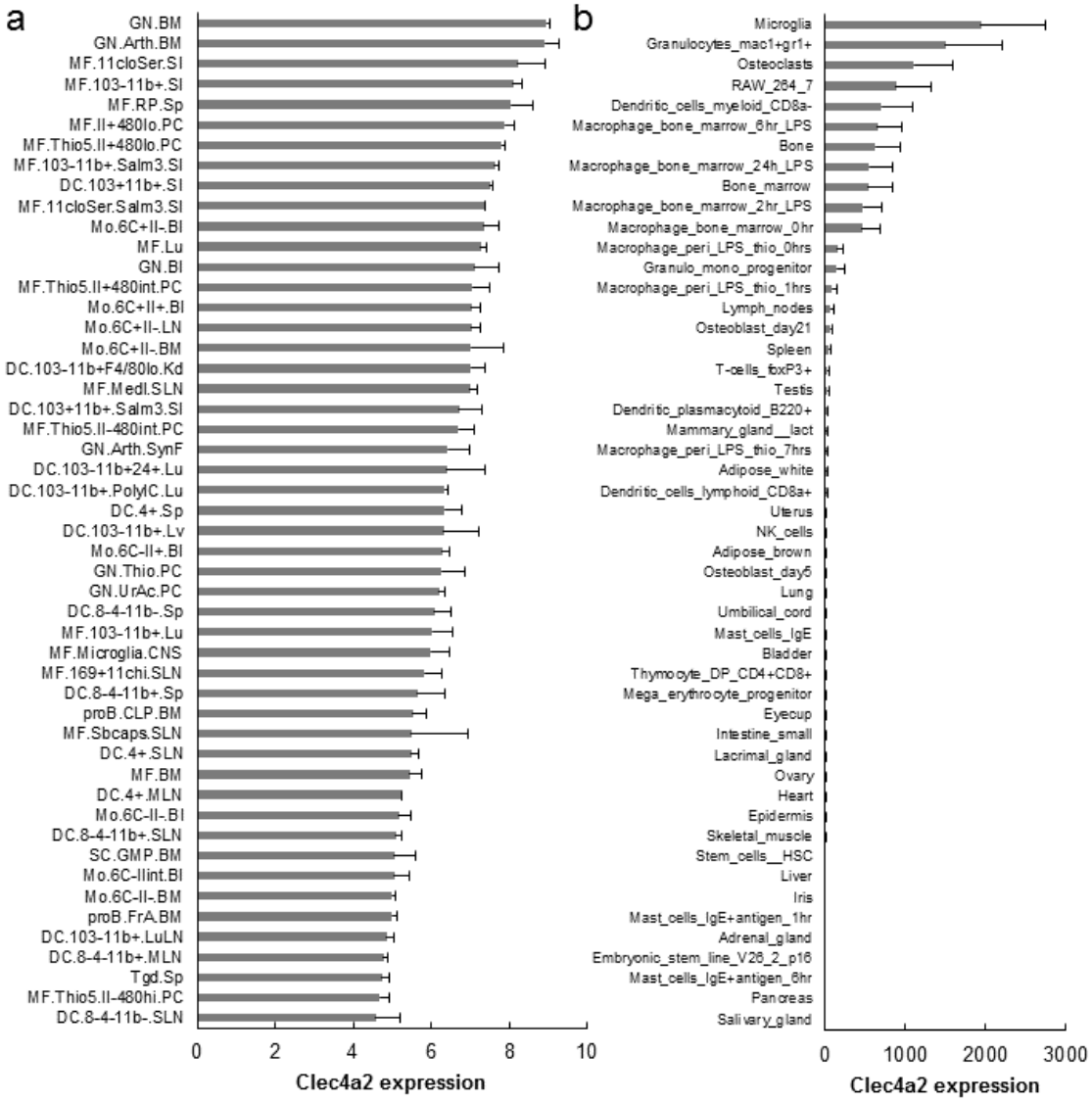

Figure 14 *Clec4a2* is expressed in the mouse immune cells and tissues mainly in granulocytes and macrophages.

These data were obtained from (a) Immunological Genome Project (http://www.immgen.org/) and (b) BioGPS (http://biogps.org) and top 50 samples are shown. (a) The whole data size is 238 samples and each data is expressed as means + SD. (b) The whole data size is 96 samples and each data is expressed as means + SE. GN: granulocyte (neutrophil), MF: macrophage, Mo: monocyte, DC: dendritic cell, Tgd: γδT cell, BM: bone marrow, SI: small intestine, Sp: spleen, Bl: blood, PC: peritoneal cavity, Lu: lung, Kd: kidney, Arth: arthritic, SynF: synovial fluid, Lv: liver, CNS: central nervous system, SLN: lymph node subcapsular, MLN: mesenteric LN, LuLN: mediastinal LN.

Figure 15

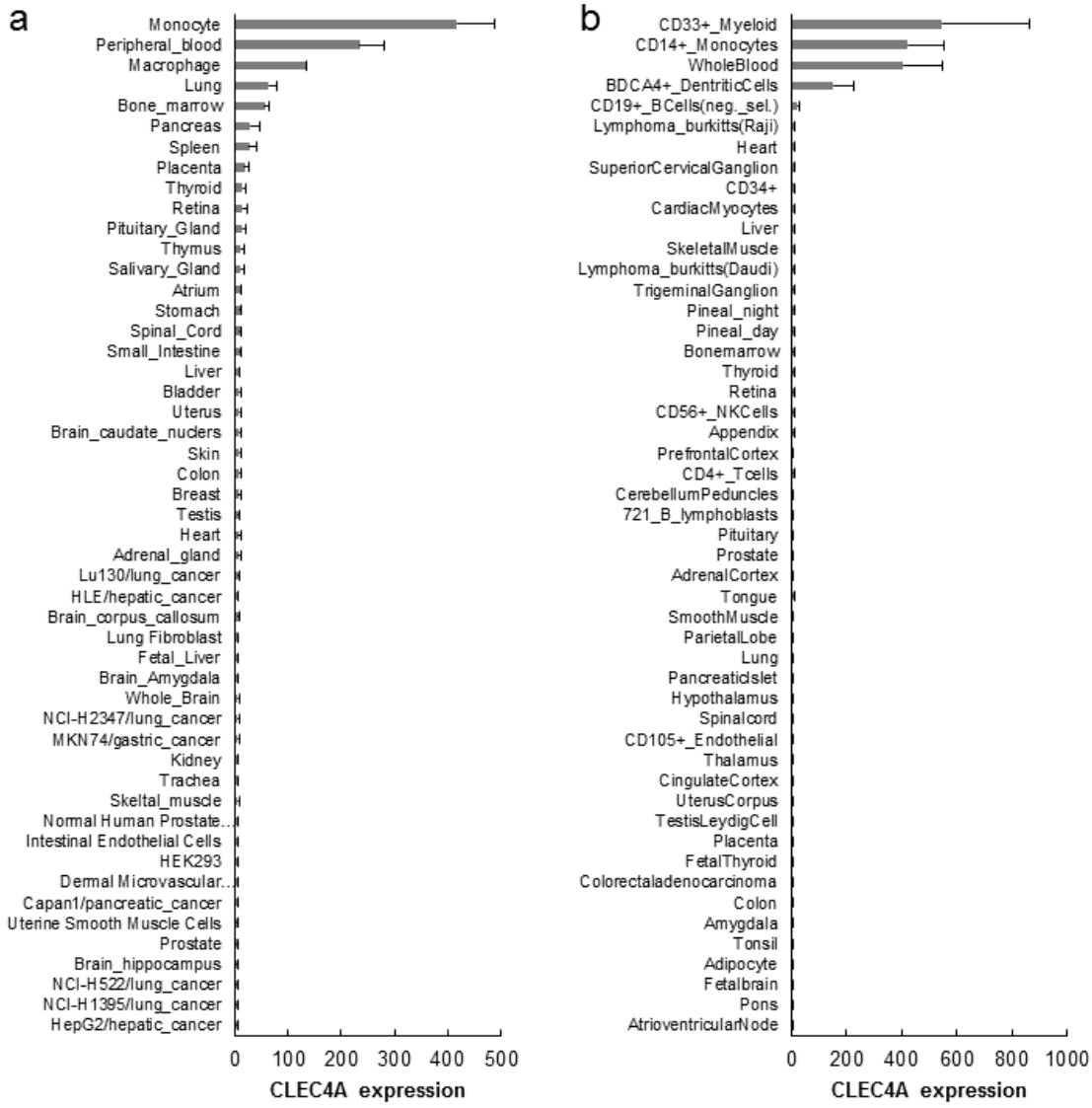

Figure 15 *CLEC4A* is expressed in the human cells and tissues mainly in monocytes and macrophages. These data were obtained from (a) Laboratory for System Biology and Medicine, at RCAST, The University of Tokyo (http://www.lsbm.org/database/) and (b) BioGPS (http://biogps.org) and top 50 samples are shown. The whole data size is (a) 108 and (b) 84 samples and each data is expressed as means + SE.

Figure 16

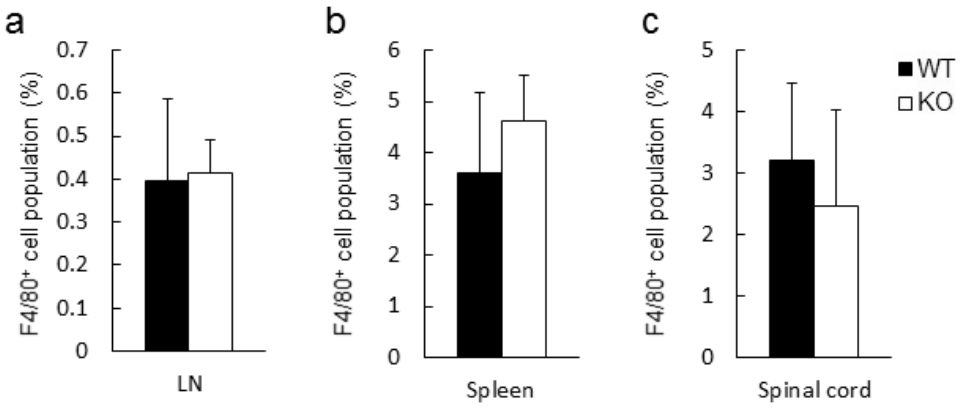

Figure 16 The population of F4/80$^+$ macrophage in draining LNs, spleen, and spinal cord of DCIR KO mice is similar to that of WT mice after immunization.

Single cell suspensions were prepared from each tissue at 28 days after immunization. Cells were stained with antibodies against F4/80 and analyzed by flow cytometry (WT, KO; n = 5 each). The data is expressed as means + SD, and obtained from one experiment.

Figure 17

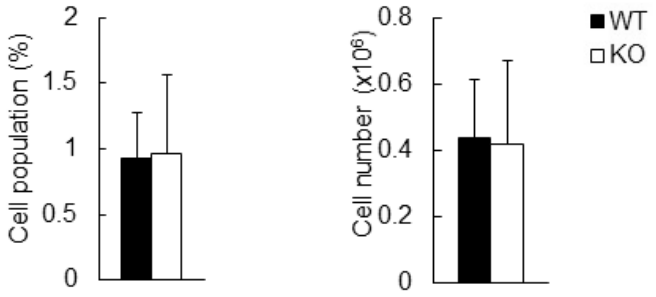

Figure 17 The number and population of F4/80^{+} macrophage in draining LNs of KO mice is comparable to that of WT mice after immunization.

Single cell suspensions were prepared from the draining LNs at 7 days after immunization. Cells were stained with antibodies against indicated antigens and analyzed by flow cytometry (WT, KO; n = 6 each). The data is expressed as means + SD, and combined result of two independent experiments.

Figure 18

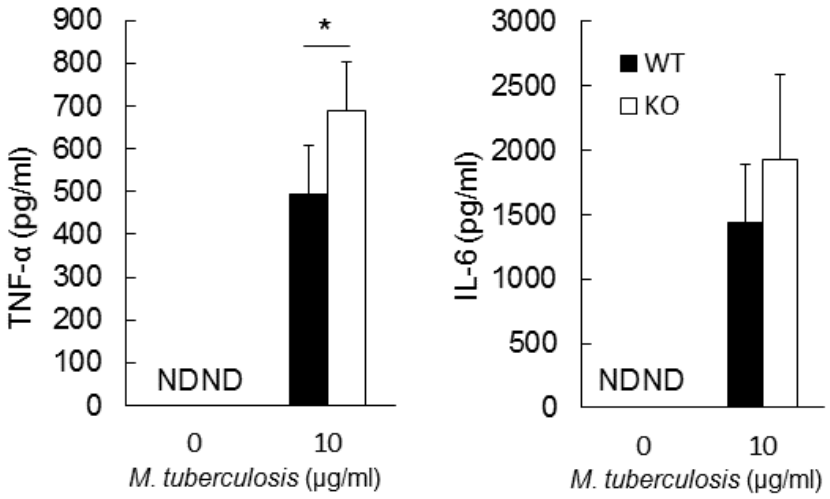

Figure 18 The *Dcir* KO macrophages produce more proinflammatory cytokines than WT cells. Macrophages were cultured for 24 hrs with MT which was used in EAE induction (WT, KO; n = 6 each). The data is expressed as means + SD, and are combined result of two independent experiments (*P < 0.05).

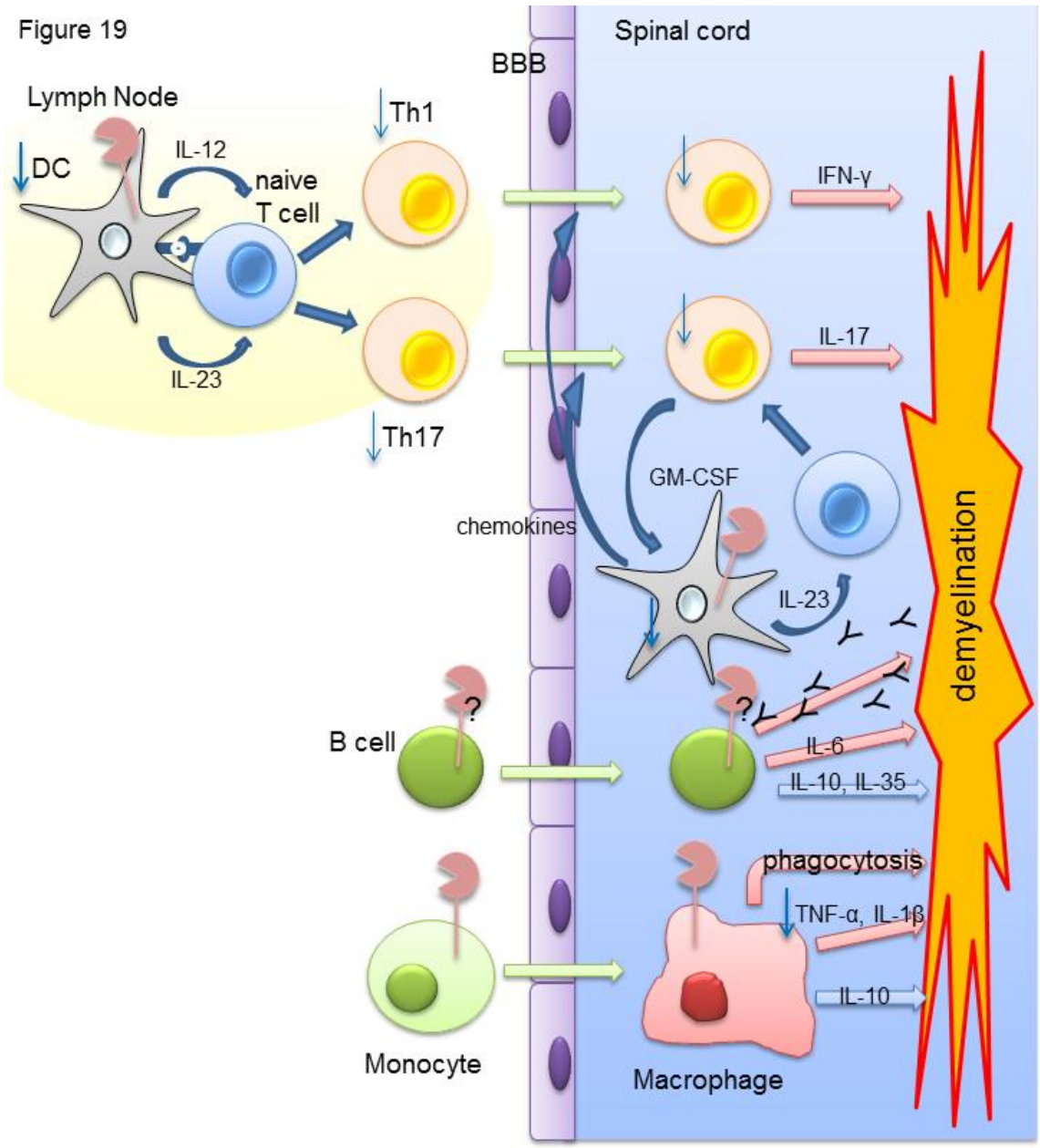

Figure 19 Possible DCIR regulation in the scheme of EAE and MS

DCIR KO mice showed severe EAE and inflammation in CNS than WT mice. In the scheme of EAE/MS presented as Fig. 3, DCIR is expressed on DCs, monocytes and macrophages. Although B cells were first reported as DCIR expression cells[190], it was not recognized in expression databases (Fig. 14, 15). The exacerbation of EAE was caused by the increase of number of DCs and T cells and the inflammatory cytokine production from macrophages. There was no difference in the antibody titer in EAE induced mice. Therefore DCIR would suppress the proliferation of DCs and the cytokine production of macrophages and regulate the development of neuroinflammation.

VII. Abbreviations

APC	antigen presenting cell
BBB	blood-brain barrier
CCR5	C-C chemokine receptor type 5
CCL3	Chemokine (C-C motif) ligand 3
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CLR	C-type lectin receptor
CNS	central nervous system
CRD	carbohydrate-recognition domain
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCIR	dendritic cell immunoreceptor
DC-SIGN	DC-specific intercellular adhesion molecule-3 grabbing nonintegrin
Dectin-1	DC-associated C-type lectin 1
EAE	experimental autoimmune encephalomyelitis
FBS	fetal bovine serum
FcRγ	Fc receptor γ
GM-CSF	granulocyte macrophage colony-stimulating factor
GM-CSFR	GM-CSF receptor
HCV	the hepatitis C virus
hDCIR	human DCIR
HE	hematoxylin and eosin
HTLV-I	human T-cell leukemia virus type I
IL	interleukin
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IFA	incomplete Freund's adjuvant
KO	knockout

LFB	Luxor fast blue
LN	lymph node
M-CSF	macrophage colony stimulating factor
mDCIR	mouse DCIR
Mincle	macrophage inducible C-type lectin
MOG	myelin oligodendrocyte glycoprotein
MR	mannose receptor
MS	multiple sclerosis
MT	*Mycobacterium tuberculosis*
NF-κB	nuclear factor-kappa B
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PRR	pattern recognition receptor
RA	rheumatoid arthritis
SD	standard deviation
SHP	*src* homology 2 domain-containing tyrosine phosphatase
STAT	signal transduction and activator of transcription
Syk	spleen tyrosine kinase
Tg	transgenic
TNF	tumor necrosis factor
Th#	type # T helper cells
WT	wild-type

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