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The Role of CCR7-Ligands in Developing Experimental Autoimmune Encephalomyelitis

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1. Introduction

Multiple sclerosis is a chronic, inflammatory, and demyelinating disease of the central nervous system characterized by the pathological infiltration of autoreactive leukocytes. Experimental autoimmune encephalomyelitis serves as a disease model for human multiple sclerosis in mouse and rat (Conlon et al., 1999). Experimental autoimmune encephalomyelitis is induced through sensitization with neuroantigens such as myelin oligodendrocyte glycoprotein that activates neuroantigen-reactive T cells in the peripheral lymphoid organs. These T cells subsequently migrate into the central nervous system and encounter endogenous neuroantigens, which reactivates them and leads to nerve demyelination. Thus, induction of encephalitogenic T cells and their migration into the central nervous system are critical for development of experimental autoimmune encephalomyelitis.

$CD4^+$ helper T cells secreting IFN-γ (Th1 cells) were long considered to be the predominant T cell subset inducing experimental autoimmune encephalomyelitis (Kuchroo et al., 2002; El-behi et al., 2010). This view was challenged by the finding that IFN-γ-deficient mice showed more severe experimental autoimmune encephalomyelitis than wild type mice (Ferber et al., 1996; Gran et al., 2002). More recently, IL-17-producing T helper cells (Th17 cells) have emerged as a critical pathogenic T cell subset causing experimental autoimmune encephalomyelitis or human multiple sclerosis (Langrish et al., 2005). Th17 cells produce the pro-inflammatory cytokines IL-17A, IL-17F, and IL-22 (Ghilardi and Ouyang, 2007), and mice lacking expression of IL-17 were resistant to the induction of experimental autoimmune encephalomyelitis (Komiyama et al., 2006). Recently, Th17 cells were demonstrated to disrupt the blood-brain barrier by the action of IL-17A (Huppert et al., 2010). Based on the many investigations on the encephalitogenic T cells, current concept is that both Th1 and Th17 cells participate in the development of EAE (El-behi et al, 2010). The

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induction of pathogenic T cells appears dependent on the coordinated migration of several cell types, a phenomena regulated by chemokines (Elhofy et al., 2002). Indeed, many chemokines have been shown to be critical for the development of experimental autoimmune encephalomyelitis (Rebenko-Moll et al., 2006). As we will discuss later, chemokines CCL19 and CCL21 regulate induction of pathogenic T cells independent of their role in the migration of immune cells. These CCR7-ligand chemokines contribute for the generation of pathogenic Th17 cells which are more efficient for the induction of experimental autoimmune encephalomyelitis.

Entry of primed T cells into the central nervous system is governed by both integrin-dependent adhesion to blood vessels and chemokine-driven migration through the blood-brain barrier. Many chemokines have been shown to be critical for the migration of activated and propagated pathogenic T cells into the central nervous system (Rebenko-Moll et al., 2006). Among them, chemokine CCL20, a ligand for CCR6, is constitutively expressed on epitherial cells of choroid plexus in mice and humans and provides ports of lymphocytes expressing a chemokine receptor CCR6 characteristic of Th17 cells (Reboldi et al., 2009). Recently, CXCL12, a ligand for CXCR7 and CXCR4, has been shown to restrict the central nervous system entry of CXCR4-expressing leukocytes, and loss of CXCL12 from abluminal surfaces of the blood-brain barrier is critical for migration of pathogenic lymphocytes into the parenchyma of the central nervous system during inducing experimental autoimmune encephalomyelitis (Cruz-Orengo et al., 2011). CCL19 and CCL21, ligands for CCR7, also have been detected at the blood-brain barrier, and suggested their involvement in CCR7-dependent lymphocyte recruitment into the central nervous system (Alt et al., 2002).

We previously identified a spontaneous mutation in mice characterized by a defect in homing of naïve T cells to the lymph node, Peyer's patches, and splenic white pulp (paucity of lymph node T cells mice; *plt/plt* mice). These mice lack the expression of CCL19 and CCL21-ser and exhibit a migration defect in T cells and dendritic cells into the T cell zone in the secondary lymphoid organs. These mice, as well as CCR7$^{-/-}$ mice, provide a good tool for the investigation of the role of these chemokines in in vivo immune response. Using *plt/plt* mouse, we have analyzed the role CCL19 and CCL21 in the regulation of immune response (Nakano et al., 1997, 1998, 2009; Gunn et al., 1999; Vassileva et al., 1999; Nakano and Gunn, 2001; Mori et al., 2001; Yasuda et al., 2007; Kuwabara et al., 2009; Aritomi et al., 2010). Unexpectedly, in vivo CD4^{+} T cell response is not decreased, but rather enhanced. When *plt/plt* mice were immunized with a protein antigen ovalbumin with complete Freund's adjuvant, both expansion of ovalbumin-responding CD4^{+} T cells in the draining lymph nodes and an in vitro recall response are prolonged and do not decline for a long time as compared with those in wild type mice.

Thus, there are two opposite possibilities; *plt/plt* mice with C57BL/6 background are resistant because of the lack of the expression of CCR7-ligands at the blood-brain barrier, or quite sensitive to the induction of experimental autoimmune encephalomyelitis because of the enhanced induction of pathogenic T cells. Using *plt/plt* mice as well as CCR7-/- mice, we investigated the role of CCR7-ligands in developing experimental autoimmune encephalomyelitis. As described below, we found *plt/plt* mice with C57BL/6 background are resistant to the induction of experimental autoimmune encephalomyelitis. This resistance is due to the failure to induce pathogenic Th17 cells because of deficient IL-23 production by dendritic cells, which results from lacking expression of CCL19 and CCL21.

2. CCL19 and CCL21 are required for the development of encephalomyelitis through generation of IL-23-dependent Th17 cells

For the development of experimental autoimmune encephalomyelitis, we used C57BL/6 wild type mouse and C57BL/6-*plt/plt* mouse. They were immunized following a standard protocol for induction of experimental autoimmune encephalomyelitis, that is, subcutaneous injection with myelin oligodendrocyte glycoprotein 35-55 peptide in complete Freund's adjuvant, and subsequent intravenous injection on day 0 and day 2 with pertussis toxin (Kuwabara et al., 2009).

2.1 *plt/plt* mouse is resistant to the induction of experimental autoimmune encephalomyelitis

When C57BL/6 mice were immunized under the standard immunization protocol as described above, wild type mice developed experimental autoimmune encephalomyelitis with 100% disease incidence with onset at day 14 and the peak at 4th week after immunization, whereas *plt/plt* mice failed to develop the disease during 42 days following immunization (Figure 1, upper left panel). Confirming CCR7-ligands requirement in the disease development, similarly treated CCR7$^{-/-}$ mice did not develop experimental autoimmune encephalomyelitis (Figure 1, upper right panel). That experimental autoimmune encephalomyelitis did not develop in *plt/plt* mice might be due to the failure of pathogenic T cells to migrate into the central nervous system because of the lack of CCR7-ligands expression, as suggested previously (Alt et al., 2002). To examine this possibility, 9 days after subcutaneous immunization draining lymph node cells from wild type mice were incubated for 3 days with myelin oligodendrocyte glycoprotein 35-55 peptide, and then CD4^{+} T cells were adoptively transferred intravenously into wild type and *plt/plt* mice. As shown in Figure 1, lower panel, both wild type and *plt/plt* recipients developed experimental autoimmune encephalomyelitis with 100% disease incidence with similar clinical scores and time courses. As expected, draining lymph node cells from immunized *plt/plt* mice did not develop experimental autoimmune encephalomyelitis in naïve wild type mice (Figure 1, lower panel). These results indicated that pathogenic T cells are able to infiltrate the central nervous system to induce experimental autoimmune encephalomyelitis despite the absence of CCR7-ligands but strongly suggest that pathogenic cells fail to be generated in *plt/plt* mice immunized with myelin oligodendrocyte glycoprotein 35-55 peptide.

Thus, the dependency of experimental autoimmune encephalomyelitis development on CCR7-ligands is not due to a defect in the migration of pathogenic T cells in *plt/plt* mice, since adoptive transfer of pathogenic CD4^{+} T cells prepared from draining lymph node cells of wild type mice results in the disease development in *plt/plt* and wild type recipient mice with similar time course and disease severity.

2.2 Deficient IL-17 and IFN-γ production by draining lymph node cells from mice lacking expression of CCR7-ligands

To examine whether pathogenic cells were generated in *plt/plt* mice, we compared the in vitro recall responses of draining lymph node cells from primed wild type and *plt/plt* mice. Draining lymph node cells were prepared 9 days after immunization when experimental

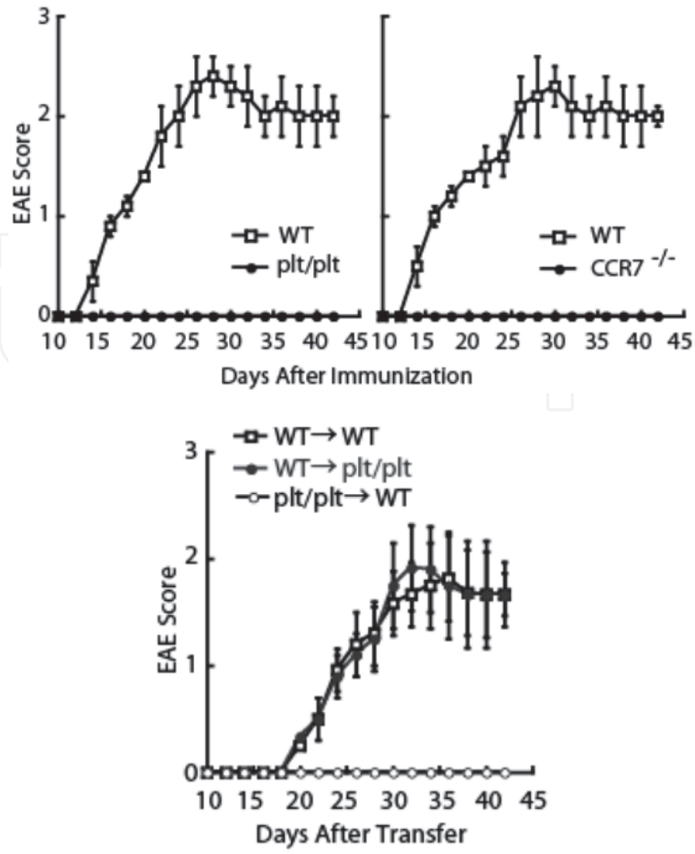

Fig. 1. Failure of *plt/plt* mice and CCR7$^{-/-}$ mice to develop experimental autoimmune encephalomyelitis. *Upper panels*, Mice were subcutaneously immunized with myelin oligodendrocyte glycoprotein 35-55 peptide in complete Freund's adjuvant at flanks and intravenously injected with pertussis toxin on days 0 and 2 (10 mice/group). Clinical symptoms were monitored for 42 days after immunization. Mean clinical score ± SD is shown. Results from wild type and *plt/plt* mice are shown in the left panel and those from wild type and CCR7$^{-/-}$ mice in the right panel. *Lower panel*, Draining lymph node cells were prepared from wild type or *plt/plt* mice 9 days after immunization and incubated with myelin oligodendrocyte glycoprotein 35-55 peptide for 3 days. Wild type CD4^{+} T cells or *plt/plt* CD4^{+} T cells ($1x10^{7}$) prepared from the treated cells were intravenously transferred into naïve and 500R X-irradiated wild type or *plt/plt* mice (10 mice/group). Results are shown as mean experimental autoimmune encephalomyelitis clinical score ± SD. WT: wild type. (Kuwabara et al., 2009)

autoimmune encephalomyelitis symptoms were not observed in wild type mice, and 14 days after immunization when the symptoms became evident. The proliferative recall responses to various doses of myelin oligodendrocyte glycoprotein 35-55 peptide were similar between draining lymph node cells from wild type and *plt/plt* mice prepared 9 days after and 14 days after immunization, suggesting T cell responses were similarly elicited in wild type and *plt/plt* mice. We also analyzed recall cytokine production to myelin oligodendrocyte glycoprotein 35-55 peptide. IL-4 and IL-10 were similarly produced by draining lymph node cells from wild type and *plt/plt* mice. Dose-dependent production of

IFN-γ or IL-17 was detected in cultures of draining lymph node cells from wild type and plt/plt mice, but production of each of these cytokines was severely diminished in *plt/plt* draining lymph node (Figure 2). These results suggest *plt/plt* T cells could be primed by immunization with myelin oligodendrocyte glycoprotein 35-55 peptide, but that the pattern of cytokine responses differed from wild type mice.

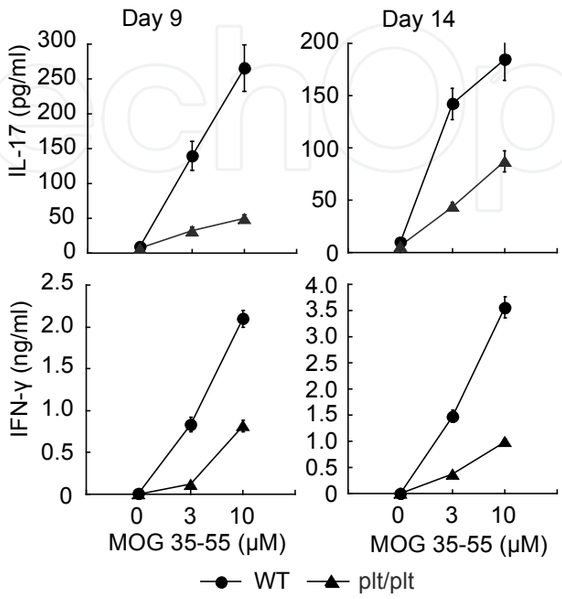

Fig. 2. *In vitro* response to myelin oligodendrocyte glycoprotein 35-55 peptide of draining lymph node cells from wild type and *plt/plt* mice. Wild type and *plt/plt* mice were immunized, as described in the legend for Figure 1. Draining lymph node cells prepared 9 or 14 days after immunization were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide at indicated doses, and assessed for IFN-γ, and IL-17 in the culture supernatants by enzyme-linked immunosorbent assay using OptEIA kits (BD Biosciences). Each result is expressed as mean ± SD. (Kuwabara et al., 2009)

2.3 Requirement for CCR-7 ligands in the generation of IL-17- or IFN-γ-secreting T cells

Reduced in vitro IL-17 and IFN-γ production by draining lymph node cells from *plt/plt* mice suggested a defect in Th17 and Th1 cell generation. To examine this possibility, draining lymph node cells were prepared 9 days after immunization, incubated with myelin oligodendrocyte glycoprotein 35-55 peptide and assessed for intracellular IL-17 or IFN-γ staining. As shown in Figure 3, $CD4^+IL\text{-}17^+$ Th17 cells were found at a much lower frequency in draining lymph node cells from *plt/plt* mice than in those from wild type mice (0.4% vs. 4.2%). Addition of CCL19 or CCL21 to DLN cells from *plt/plt* mice during incubation with myelin oligodendrocyte glycoprotein 35-55 peptide restored Th17 cell generation from 0.4% to 3.0 or 4.1%, respectively (Figure 3). Also the frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 cells was much lower in *plt/plt* mice than in WT mice (0.4% vs. 4.4%). Addition of CCL19 or CCL21 restored Th1 cell generation in *plt/plt* mouse draining lymph node cells from 0.4% to 3.1 or 3.2%, respectively (Figure 3). These results support the hypothesis that the defect in generating Th17 or Th1 cells in *plt/plt* mice was due to the lack of CCR7-ligand expression.

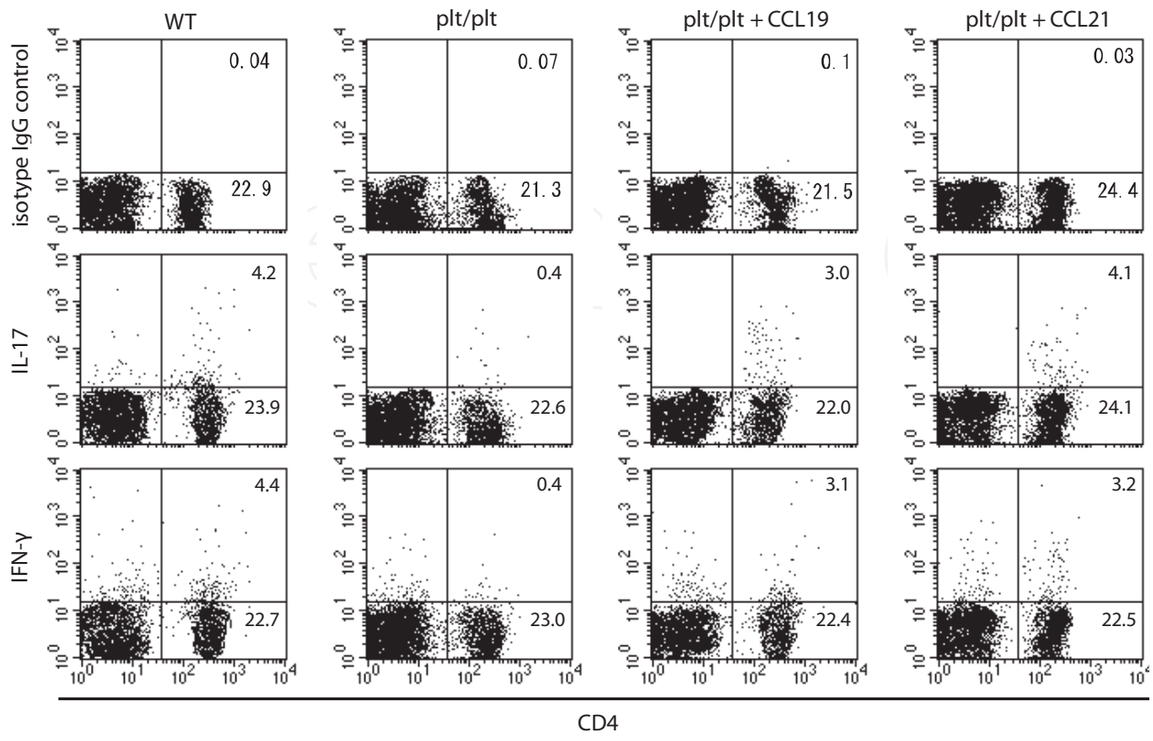

Fig. 3. Analysis of the T cell response in draining lymph nodes from wild type and *plt/plt* mice immunized for experimental autoimmune encephalomyelitis induction and generation of Th17 or Th1 cells by CCR7-ligand. Draining lymph node cells were prepared from wild type and *plt/plt* mice 9 days after immunization as described in the legend for Figure 1. Draining lymph node cells were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide in the presence or absence of CCL21 or CCL19 (100ng/ml) then assessed for intracellular IL-17 or IFN-γ expression on a flow cytemeter. Numbers in right quadrants are the percentage to the total cells. (Kuwabara et al., 2009)

2.4 Decreased production of IL-12 and IL-23 by draining lymph node cells from *plt/plt* mice

For the optimal induction of IL-17-producing cells, IL-6, TGF-β and IL-23 are required (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006). IL-12 is critical for inducing IFN-γ-producing cells (Seder and Paul, 1994) . Deficient production of IL-17 and IFN-γ suggested that these cytokines were insufficiently produced in draining lymph node cells from *plt/plt* mice. Draining lymph node cells prepared from wild type and *plt/plt* mice 4 or 9 days after immunization, similar levels of IL-6 and TGF-β production were observed following incubation with myelin oligodendrocyte glycoprotein 35-55 peptide. In contrast, as shown in Figure 4, the expression of IL-23p19 mRNA and IL-12p35 mRNA and production of IL-23 and IL-12 were much lower in cells from *plt/plt* mice than wild type mice, suggesting that the defect in production of IL-17 and IFN-γ in draining lymph node cells from *plt/plt* mice was due to insufficient provision of IL-23 and IL-12.

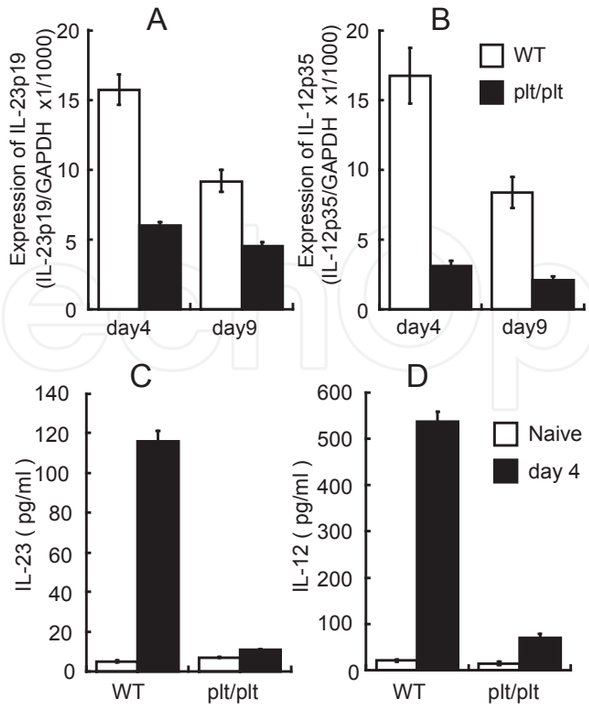

Fig. 4. Severely impaired production of IL-12 and IL-23 in draining lymph node cells from *plt/plt* mice. Draining lymph node cells were prepared 4 or 9 days after immunization as described in the legend for Figure 1. **A, B,** Expression of IL-23p19 mRNA (A) and IL12p35 mRNA (B) in $CD11c^+$ cells was estimated by quantitative RT-PCR in draining lymph node cells from wild type and *plt/plt* mice. The expression is shown as mean ± SD of the ratio to GAPDH, an internal control. These experiments were repeated 5 times with similar results. **C, D,** Draining lymph node cells from naïve mice or 4days after immunization were incubated with 10 μM myelin oligodendrocyte glycoprotein 35-55 peptide for 24 hrs. Culture supernatants were assessed for IL-23 (C) and IL-12 (D). Results of triplicate assay were presented as mean ± SD. (Kuwabara et al., 2009)

2.5 Th17 cells critically participate in the development of experimental autoimmune encephalomyelitis

Previous reports demonstrated that neuroantigen-specific Th17 or Th1 cell is responsible for experimental autoimmune encephalomyelitis induction (Langrish et al., 2005; Lees et al., 2008; Kroenke et al., 2008). To determine which defect in generating Th17 or Th1 cells was more critical in the resistance to experimental autoimmune encephalomyelitis development, draining lymph node cells from plt/plt mice were stimulated in vitro with myelin oligodendrocyte glycoprotein 35-55 peptide under the conditions for generating Th17 cells or Th1 cells, enriched for $CD4^+$ T cells, and transferred into wild type mice. As shown in Figure 5, $CD4^+$ T cells containing Th17 cells ($CD4^+IL\text{-}17^+$cells: 9.2%, $CD4^+IFN\text{-}\gamma^+$ cells: 0.1%) induced experimental autoimmune encephalomyelitis in the recipient mice with 100% disease incidence, whereas those containing Th1 cells ($CD4^+IL\text{-}17^+$cells: 0.1%, $CD4^+IFN\text{-}\gamma^+$ cells: 11.0%) did not, indicating that Th1 cells are less efficient at inducing experimental autoimmune encephalomyelitis, at least under the conditions employed. The cell

preparation containing Th17 or Th1 cells was confirmed to predominantly produce IL-17 or IFN-γ, respectively. The cells similarly prepared from WT mice and enriched for Th1 cells ($CD4^+$ $IL\text{-}17^+$cells: 0.6%, $CD4^+$ $IFN\text{-}\gamma^+$ cells: 20.3%) also failed to elicit experimental autoimmune encephalomyelitis in the recipient mice, whereas those containing Th17 cells ($CD4^+$ $IL\text{-}17^+$cells: 19.2%, $CD4^+$ $IFN\text{-}\gamma^+$ cells: 0.4%) elicited experimental autoimmune encephalomyelitis. These findings strongly support our interpretation that the defect in generating Th17 cells is crucial in the resistance to experimental autoimmune encephalomyelitis development in *plt/plt* mice under the conditions employed.

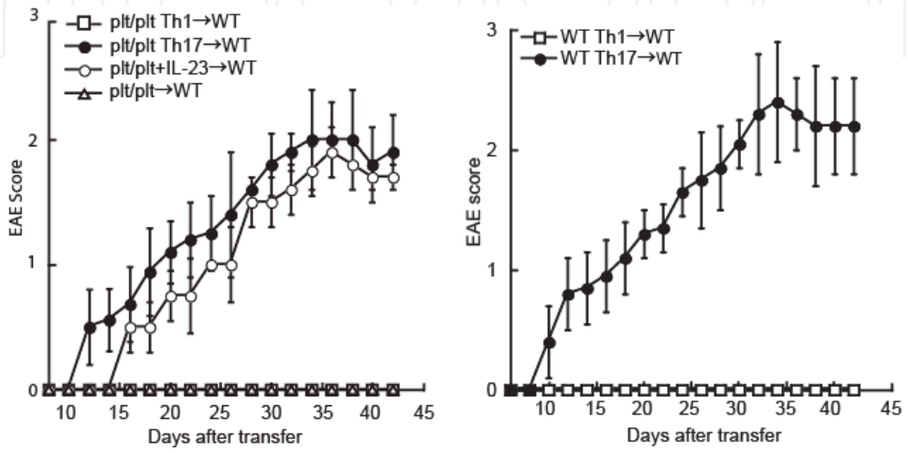

Fig. 5. Th17-enriched, rather than Th1-enriched, cell population was responsible for experimental autoimmune encephalomyelitis development in recipient mice. Draining lymph node cells from primed *plt/plt* (left panel) or wild type (right panel) mice were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide for 3 days in the presence of CCL19, IL-12 and anti-IL-4 and anti-IL-23 mAbs for developing Th1 cells, in the presence of CCL19, IL-23 and anti-IL-4 and anti-IFN-γ mAbs for developing Th17 cells, or in the presence of IL-23 alone. $CD4^+$ T cells ($1x10^7$) prepared from the treated cells were intravenously transferred into naïve and 500R X-irradiated wild type mice (10 mice/group). Experimental autoimmune encephalomyelitis development is shown as a mean clinical score ± SD. (Kuwabara et al., 2009)

2.6 IL-23-dependent induction of encephalitogenic Th17 cells

Deficient IL-23 production in draining lymph node cells from *plt/plt* mice prompted us to evaluate the role of IL-23 in inducing Th17 cells. Addition of exogenous IL-23 to $CD4^+$ draining lymph node cells from immunized *plt/plt* mice stimulated with immobilized anti-CD3 and anti-CD28 mAbs increased the frequency of Th17 cells from 0.18% to 1.34%, supporting the idea that the defect in developing Th17 cells in *plt/plt* mice was due to reduced production of IL-23. To confirm that stimulation with IL-23 was able to induce pathogenic T cells in experimental autoimmune encephalomyelitis induction, draining lymph node cells from immunized *plt/plt* mice were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide in the presence of IL-23, enriched for $CD4^+$ T cells, and adoptively transferred into naïve wild type mice, which resulted in the development of experimental autoimmune encephalomyelitis in the recipient mice (Figure 5, left panel). These results suggested that exogenous IL-23 was able to stimulate *plt/plt* mouse draining

lymph node cells along with myelin oligodendrocyte glycoprotein 35-55 peptide to induce pathogenic Th17 cells, consistently, with the critical role of IL-23 in the induction phase of experimental autoimmune encephalomyelitis (Thankker et al., 2007). Taken all together, these findings suggest that the defect in *plt/plt* mice is likely a defect in Th17 cell generation due to deficient IL-23 production.

2.7 CCR7-ligands stimulate dendritic cells to produce IL-23

Dendritic cells are known to produce IL-23 (Oppmann et al., 2000). The reduced production of IL-23 in the incubation of *plt/plt* draining lymph node cells with myelin oligodendrocyte glycoprotein 35-55 peptide suggests the dependency of the IL-23 production on CCR7-ligands. To confirm this possibility, we prepared bone marrow-derived dendritic cells and stimulated the cells with CCR7-ligands or other chemokines. Lipopolysaccharide was used as a positive control for induction of IL-23p19 mRNA (Oppmann et al., 2000). CCL19 or CCL21 increased IL-23p19 mRNA expression, although not to the same extent as lipopolysaccharide (Figure 6-A, left and middle panels). The chemokines CCL5 and CXCL12 did not stimulate bone marrow-derived dendritic cells to produce IL-23 (Figure 6, left panel). Confirming that CCL19 and CCL21 stimulate DCs through CCR7 to express IL-23p19mRNA, bone marrow-derived dendritic cells from CCR7$^{-/-}$ mice did not respond to the chemokines (Figure 6-A, right panel).

Draining lymph node cells also express IL-23p19 mRNA in response to CCR7-ligands. Draining lymph node cells from immunized wild type, *plt/plt*, or CCR7$^{-/-}$ mice were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide for 6 hours in the presence or absence of CCL19 or CCL21. Then, CD11c^{+} cells were enriched and assayed for IL-23p19 mRNA expression. As shown in Figure 6-B, left panel, CD11c^{+} cells from wild type mice expressed much higher IL-23p19 mRNA than those from naïve mice, and addition of CCL19 did not further enhance IL-23p19 mRNA expression in these cells from immunized wild type mice, probably because they had been exposed to CCL19 produced in draining lymph nodes. In CD11c^{+} cells from *plt/plt* mice, however, addition of exogenous CCL19 or CCL21 increased IL-23p19 mRNA expression (Figure 6-B, middle). As expected, cells from CCR7$^{-/-}$ mice did not respond to the addition of CCR7-ligands (Figure 6-B, right panel).

CCR7-ligands also stimulated IL-23 production by bone marrow-derived dendritic cells from wild type and *plt/plt* mice and by draining lymph node cells from *plt/plt* mice (Figure 6-C, D). Draining lymph node cells alone from immunized wild type mice produced much more IL-23 than those from naïve wild type mice, probably because endogenous CCR7-ligands induced sufficient level of IL-23 production (Figure 6- C, D). Taken together, the results shown in Figure 6 demonstrate that CCL19 or CCL21 is necessary and sufficient to induce IL-23 production from dendritic cells. Confirming that IL-23 production in response to a CCR7-ligand plays a critical role in Th17 induction, in a dose-dependent fashion anti-IL-23 mAb inhibited Th17 cell generation following incubation of draining lymph node cells from *plt/plt* mice with myelin oligodendrocyte glycoprotein 35-55 peptide in the presence of CCL19 or CCL21 (Figure 7).

Also in vivo expression of IL-23 in dendritic cells was observed in the presence of CCR7-ligands, but not in the absence of them. When mice were immunized subcutaneously with a protein antigen ovalbumin in complete Freund's adjuvant, expression of IL-23p19 mRNA

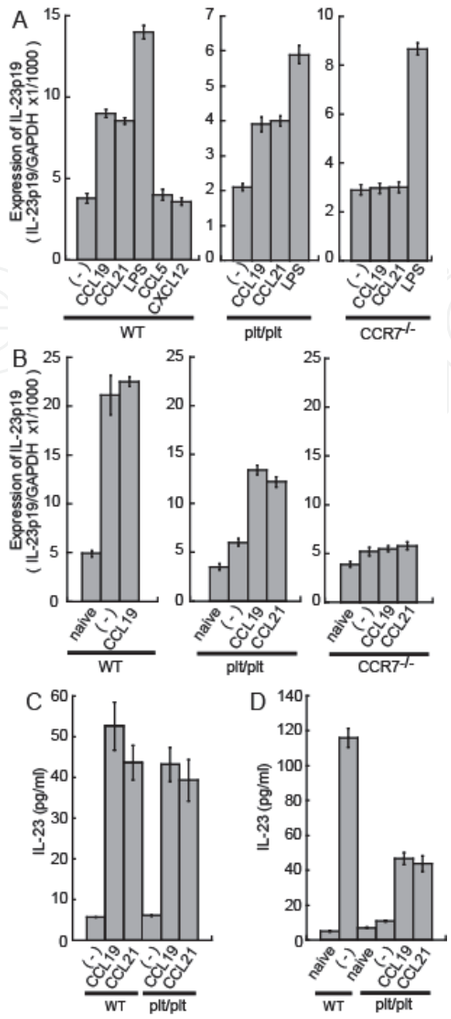

Fig. 6. CCR7-ligands stimulate dendritic cells to express IL-23p19 mRNA and to produce IL-23. **A,** Bone marrow-derived dendritic cells were prepared from wild type, *plt/plt*, and CCR7$^{-/-}$ mice, and stimulated with lipopolysaccharide or indicated chemokines at 100ng/ml for 6 hours. Cellular RNA was prepared from each cell population and IL-23p19 mRNA expression was evaluated by quantitative RT-PCR. The expression is shown as mean ± SD of the ratio to GAPDH, an internal control. **B,** Draining lymph node cells were prepared 4 days after immunization from wild type, *plt/plt*, and CCR7$^{-/-}$ mice, and incubated with 10μM myelin oligodendrocyte glycoprotein 35-55 peptide in the presence or absence of CCL19 or CCL21 for 6 hrs. CD11c^{+} cells were enriched with a positive selection kit (BD Biosciences) by MACS. CD11c^{+} cells were 89.2%, 92.2%, and 90.4% for wild type, *plt/plt*, and CCR7$^{-/-}$ mice, respectively. Cellular RNA was prepared from each cell population and assessed for IL-23p19 mRNA expression by quantitative RT-PCR. Controls were lymph node cells from naïve mice. Expression is shown as mean ± SD of the ratio to GAPDH as an internal control. **C, D,** Bone marrow-derived dendritic cells (C) or draining lymph node cells (D) from wild type and *plt/plt* mice were stimulated as described above for 24 hours. The supernatants were assessed for IL-23 using an enzyme-linked immunosorbent assay kit (BD Biosciences). Results are shown as a mean ± SD of triplicate assay. (Kuwabara et al., 2009)

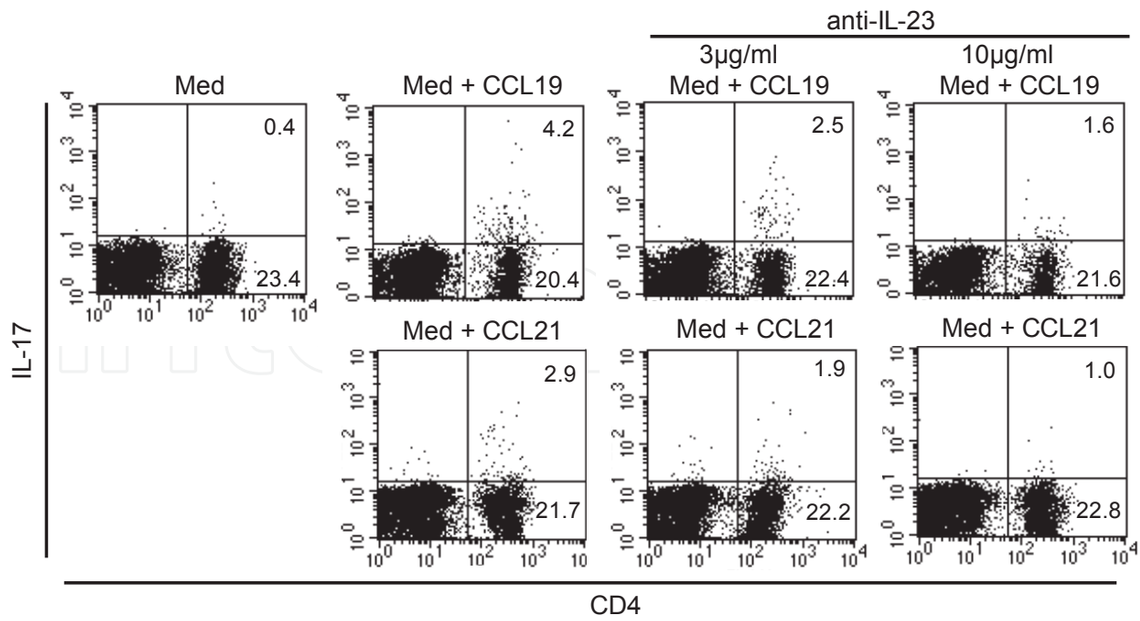

Fig. 7. Draining lymph node cells from *plt/plt* mice were incubated with myelin oligodendrocyte glycoprotein 35-55 peptide for 2 days in the presence or absence of CCL21 or CCL19 alone or with anti-IL23 mAb. The cells were analyzed for CD4 expression and intracellular IL-17. (Kuwabara et al., 2009)

and its protein was much higher in the draining lymph nodes CD11c$^+$ dendritic cells from wild type mice than in those from *plt/plt* mice. Thus, CCR7-ligands are required for IL-23 production both in vivo and in vitro.

IL-12p35 mRNA expression and IL-12 production in bone marrow-derived dendritic cells from *plt/plt* mice were also induced by the addition of exogenous CCL19 or CCL21.

It was also possible CCR7-ligands directly stimulated CD4$^+$ T cells to produce IL-17. However, this seemed unlikely since CD4$^+$ T cells isolated from naïve *plt/plt* mice or *plt/plt* mice primed with myelin oligodendrocyte glycoprotein 35-55 peptide were not induced to produce IL-17 in response to immobilized anti-CD3 and anti-CD28 mAbs in the presence of exogenously added CCL19 or CCL21. We concluded CCR7-ligands stimulated dendritic cells to produce IL-23, which in turn resulted in Th17 differentiation. Consistently, IL-23 has been shown to be a critical Th17 growth, survival and pathogenesis-inducing factor (Verdhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006; Ghoreschi et al., 2010).

2.8 CCR7-ligands promote the generation of pathogenic Th17 cells

To determine the pathogenicity of draining lymph node T cells from *plt/plt* mice that had been incubated with CCR7-ligands under experimental autoimmune encephalomyelitis inducing conditions, 9 days after immunization cells were incubated for 3 days with myelin oligodendrocyte glycoprotein 35-55 peptide in the presence of CCL19 or CCL21. CD4+ T cells were enriched from the treated cells and intravenously transferred into naïve wild type mice. As shown in Figure 8, the recipient mice developed experimental autoimmune encephalomyelitis with more than 70% disease incidence.

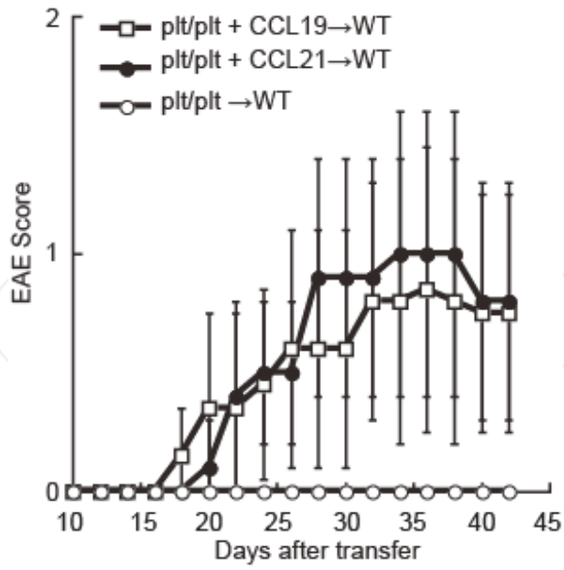

Fig. 8. Restoration of pathogenic T cells by incubation with CCR7-ligands. Draining lymph node cells from immunized *plt/plt* mice ($CD45.2^+$) were incubated at $4x10^6$ cells/ml with 10 μM myelin oligodendrocyte glycoprotein 35-55 peptide in the presence of CCR7-ligands (100 ng/ml) for 3 days. $CD4^+$ T cells ($3x10^7$) prepared from the treated cells were intravenously transferred into naïve and 500R X-irradiated wild type mice, and mice were monitored for experimental autoimmune encephalomyelitis (10 mice/group). A mean ± SD of experimental autoimmune encephalomyelitis clinical score is plotted. The experimental autoimmune encephalomyelitis incidence was 0% for recipients of cells incubated in the absence of CCR7-ligands, 70% for those in the presence of CCL19, 80% for those in the presence of CCL21. (Kuwabara et al., 2009)

2.9 CCR7 ligands up-regulate IL-23 through PI3-kinase and NF-κB pathway in dendritic cells

Finally, we explored the molecular mechanism involved in CCR7-ligand-induced IL-23 production in dendritic cells, using $CD11c^+$ spleen and bone marrow-derived dendritic cells. Although IL-23 is a heterodimeric molecule of a p40 subunit and a p19 subunit, p19 expression is the rate-limiting factor for IL-23 production (Oppmann et al., 2000). Several reports have shown that MAPK and PI3K/Akt signaling pathways triggered by CCR7 activation modulate dendritic cell function (Yanagawa & Onoe, 2002, 2003; Sanchez-Sanchez et al., 2004; Iijima et al., 2005; Riol-Blanco et al., 2005). Similar to previous studies, stimulation of dendritic cells with CCL19 or CCL21 resulted in the activation of Erk1/2, JNK, p38 MAP kinase and PI3K (Kuwabara et al., 2011). The CCR7 ligand-induced increase in IL-23 p19mRNA transcription was markedly antagonized only by a PI3K inhibitor. In contrast, the ability of dendritic cells to migrate toward CCL19 or CCL21 was not blunted by the PI3K inhibitor, indicating that signaling pathways triggered by CCR7 for IL-23 production and for migration are different (Kuwabara et al., 2011).

PI3K/Akt activation is known to induce NF-κB activation (Kane et al., 1999). Lipopolysaccharide activates NF-κB in dendritic cells to produce IL-23 (Utsugi, et al., 2006; Mise-Omata, et al.,2007; Varmody, et al., 2007; Liu, et al., 2009). We examined if NF-κB

activation was also critical for CCR-7-mediated IL-23 production. When dendritic cells were stimulated with CCL19 or CCL21, translocation of NF-κB was observed from the cytoplasm into the nucleus. IκBα is an NF-κB inhibitor whose levels are inversely and closely correlated to the activation of NF-κB (Karin & Ben-Nerah). We found stimulation of dendritic cells with CCL19 or CCL21 degraded IκBα, which was prevented by inhibition of PI3K/Akt signaling. In addition, NF-κB inhibitors blunted the ability of CCR7 ligands to induce IL-23 production. Inhibition of PI3K activation abolished CCR7 ligand-mediated NF-κB DNA binding activities (Kuwabara et al., 2011). Thus, CCR7 ligands triggers NF-κB activation through PI3K/Akt signaling, which results in the production of IL-23. It was also confirmed that CCR7 ligand–stimulated dendritic cells induce Th17 cells as antigen presenting cells.

3. Conclusions

We have investigated the role of CCR7-ligands, CCL19 and CCL21, in the development of experimental autoimmune encephalomyelitis, a disease model for human multiple sclerosis in mice. For this aim we used *plt/plt* mouse lacking expression of CCL19 and CCL21-ser, which we previously identified. These mice are resistant to the induction of experimental autoimmune encephalomyelitis under the standard protocol. In these mice encephalitogenic Th17 cells are not generated. For the generation of Th17 cells IL-23 is required but dendritic cells in these mice are unable to produce IL-23. CCR7 ligands stimulate dendritic cells to produce IL-23, and dendritic cells treated with CCR7 ligands are able to generate Th17 cells as antigen-presenting cells. The molecular mechanism involved in CCR7 ligand-induced IL-23 production in dendritic cells was analyzed. CCR7 ligands trigger PI3K/Akt signaling pathway in dendritic cells through CCR7 and activate NF-κB, which results in the production of IL-23. The signaling pathway for IL-23 production is different from that for migration toward CCR7 ligands. For the development of strategies to treat experimental autoimmune encephaslomyelitis or human multiple sclerosis, we have to elucidate precise mechanisms for IL-23 production in dendritic cells through CCR7 and how dendritic cells are stimulated with CCR7 ligands in vivo to produce IL-23.

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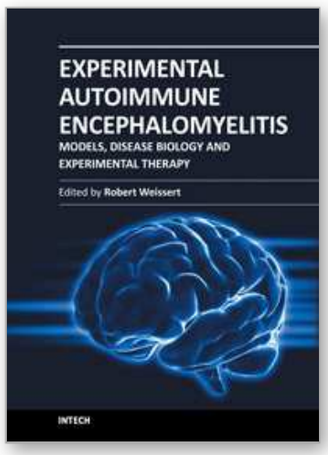

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Experimental Autoimmune Encephalomyelitis - Models, Disease Biology and Experimental Therapy is totally focused on the model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). The book chapters give a very good and in depth overview about the currently existing and most used EAE models. In addition, chapters dealing with novel experimental therapeutic approaches demonstrate the usefulness of the EAE model for MS research. With an international perspective, this book features contributions from authors throughout the world, Australia, Germany, Japan, Spain, Taiwan, and USA. There is an impressive international Faculty that provides insight into current research themes. This further demonstrates the importance of EAE in research all over the world. The book will provide established researchers and students with novel insights and guidance for their research and will help to push the field forward.

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