## 1. Introduction

Invariant NKT (iNKT) cells are regulatory T lymphocytes reactive to lipid antigen presented by a monomorphic glycoprotein CD1d (Bendelac et al., 2007; Kronenberg, 2005; Taniguchi et al., 2003). Many previous reports have documented that the number or function of iNKT cells is altered in patients with autoimmune diseases such as multiple sclerosis (MS) (Araki et al., 2003; Illés et al., 2000; Kojo et al., 2001; van der Vliet et al., 2001b; Wilson et al., 1998). Studies using transgenic mice that over- or under-express iNKT cells have basically supported the involvement of iNKT cells in the pathogenesis of autoimmune diseases (Miyake and Yamamura, 2007a). Moreover, stimulating iNKT cells with synthetic glycolipids has proven effective for preventing experimental autoimmune encephalomyelitis (EAE) (Miyamoto et al., 2001; Pál et al., 2001) or spontaneous type 1 diabetes (T1D) in NOD mice (Naumov et al., 2001; Sharif et al., 2001), indicating the important role of iNKT cells in controlling pathogenic autoreactivity and maintaining immune homeostasis (Miyake and Yamamura, 2007b). However, more recent studies have shown that iNKT cells may augment inflammatory conditions in models of arthritis (Chiba et al., 2005; Kim et al., 2005; Ohnishi et al., 2005), $CD8^+$ T cell-mediated diabetes (Griseri et al., 2005), experimental colitis (Ronet et al., 2005; Ueno et al., 2005) and airway hypersensitivity reactions (Akbari et al., 2003; Meyer et al., 2007). These results indicate that unlike $CD4^+$ $CD25^+$ regulatory T cells that appear to be a faithful regulator of unwanted immune responses (Sakaguchi and Sakaguchi, 2005), iNKT cells' help is only conditional and would occasionally take part in augmentation of harmful inflammation. How activation of iNKT cells manifests such opposing results and what is an initial trigger for the regulatory iNKT cell responses has remained to be unanswered. Here we review recent advances in the research of iNKT cells that may be relevant for understanding the "Janus-like" behavior of iNKT cells (Wilson and Delovitch, 2003). Our ultimate goal is to seek ways for making iNKT cells serve as a reliable guardian for our health.

## 2. General properties of iNKT cells

Although iNKT cells express T cell receptor (TCR) α- and β-chains, their TCR diversity is very limited owing to their expression of a single α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in human) coupled with a β-chain rearranged with a limited Vβ gene segments (Vβ8.2, Vβ2 and Vβ7 in mice, Vβ11 in human). Unlike conventional T cells, they constitutively express memory/activated T cell phenotype and are capable of producing enormous amounts of pro- and anti-inflammatory cytokines shortly after TCR engagement (Bendelac et al., 2007; Kronenberg, 2005; Miyake and Yamamura, 2005; Taniguchi et al., 2003). The cytokine burst following iNKT cell activation then triggers a maturation process in downstream cells such as NK cells, dendritic cells (DCs), B cells and T cells, leading to subsequent alteration of a broad range of adaptive immune responses. It is widely accepted that they could behave very much like innate lymphocytes rather than conventional T cells (Mempel et al., 2002), and owing to the rapidity with which they respond to various stimuli, they play an important role in bridging innate and adaptive arms of immune response.

The ability of iNKT cells to produce regulatory cytokines is so outstanding that they could efficiently alter an adaptive immune response. Mouse iNKT cells can produce interferon-γ (IFN- γ), IL-2 (Jiang et al., 2005), -3 (Leite-de-Moraes et al., 2002), -4, -5, -13, -17, -21 (Coquet et al., 2007), GM-CSF (Leite-de-Moraes et al., 2002), and osteopontin (Diao et al., 2004) after an optimal engagement of TCR. However, it does not mean that iNKT cells would purposefully use all the listed cytokines. In fact, it can be assumed that except for extreme conditions (like stimulation with strong agonists), iNKT cells may produce only a set of Th1 or Th2 cytokines in physiological conditions. We support this postulate because the TCR engagement by an endogenous ligand is likely to be modest or suboptimal in most situations (Sakuishi et al., 2007). With regard to their role in balancing immune homeostasis, an organized production of Th1, Th2 or Th17 cytokines is probably required for iNKT cells to conduct meaningful jobs.

## 3. Exogenous glycolipids stimulatory for iNKT cells

Since a marine sponge-derived glycosphingolipid, α-galactosylceramide (α-GalCer), was discovered as a potent ligand for iNKT cells (Kawano et al., 1997), a synthetic α-GalCer has widely been used for study of iNKT cells as a surrogate ligand (Fig. 1). It is now established that two lipid chains of α-GalCer are inserted to hydrophobic grooves of the CD1d glycoprotein expressed by antigen presenting cells (APCs) (McCarthy et al., 2007), whereas the α-linked sugar moiety is accessible and recognized by the TCR of iNKT cells. Recently, the crystal structure of the invariant TCR and CD1d loaded with α-GalCer has shown a very unique orientation of TCR towards CD1d (Borg et al., 2007), which allows a selective involvement of the invariant α-chain for recognition of the α-linked sugar.

Comparison of α-GalCer with its structurally altered analogues has provided important insights into how iNKT cells may differentially respond to glycosphoingolipids with lipid tail variants (Brutkiewicz, 2006; Miyake and Yamamura, 2007b). As a representative example, we showed previously that an α-GalCer analogue called OCH (Miyamoto et al., 2001; Oki et al., 2004, 2005), with a shorter sphingosine chain (Fig. 1), would selectively stimulate IL-4 production from iNKT cells, whereas α-GalCer stimulation induces both IL-4 and IFN-γ. Accordingly, OCH stimulation of iNKT cells favors a Th2 bias of immune responses *in vivo*, as compared to α-GalCer stimulation.

α-linked sugars such as α-GalCer are not recognized as a product of mammalian cells, implying that α-GalCer is not a physiological ligand for iNKT cells. Currently, it is well recognized that iNKT cells can be activated during infectious diseases (Tupin et al., 2007). Interestingly, it has been reported that α-GalCer-like glycosphingolipids are rather ubiquitously found in the environment, indicating that α-GalCer may be actually derived from bacteria residing with the marine sponge. Whether or not α-GalCer is derived from bacteria, we may ask a number of questions as to whether infectious diseases may influence on autoimmune disease via activation of iNKT cells

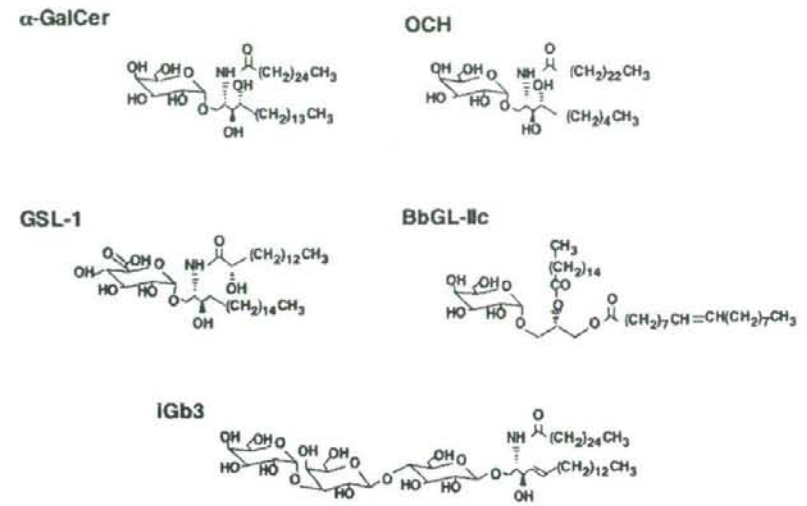


Fig. 1. Structure of glycolipid ligands for iNKT cells. Shown here are the structure of NKT cell agonists: α-galactosylceramide (α-GalCer) (Kawano et al., 1997), an α-GalCer analog called OCH, bearing a shorter sphingosine chain (Miyamoto et al., 2001). *Sphingomonas*-derived glycosphingolipid GSL-1 (Kinjo et al., 2005). *Borrelia burgdorferi*-derived diacylglycerol glycolipid BbGL-IIc (Kinjo et al., 2006), and isoglobotryhexosylceramide (iGb3) (Zhou et al., 2004).

(Godfrey and Berzins, 2006). Although multiple pathways are operative for iNKT cell activation in facing microbial challenge, it has been shown that glycosphingolipids from LPS-negative α-Proteobacteria such as *Sphingomonas* (Fig. 1) could stimulate a proportion of iNKT cells (Kinjo et al., 2005; Mattner et al., 2005). They also found that diacylglycerol glycolipids, extracted from *Borrelia burgdorferi*, stimulate at least 25% of iNKT cells (Kinjo et al., 2006; Kinjo et al., 2005). It is currently thought that arthritis and carditis found in Lyme disease following *B. burgdorferi* infection may be mediated by an autoimmune process. Whether iNKT cells activated by the diacylglycerol lipids may contribute to the pathogenesis of Lyme disease is an interesting question to be addressed. Likewise, an interesting idea is that relapse of MS following infection may be triggered by iNKT cells that are activated in response to microbial stimuli. Of note is that iNKT cells may produce osteopontin, which is reported to trigger relapses of EAE by promoting the survival of activated T cells in the inflammatory site (Hur et al., 2007).

## 4. Endogenous ligand for iNKT cells: search is not over

Search for an endogenous ligand of iNKT cells has led to the identification of lysosomal glycosphingolipid isoglobotryhexosylceramide (iGb3) (Fig. 1), a β-linked sugar capable of stimulating iNKT cells as a potential endogenous ligand for mouse and human iNKT cells (Mattner et al., 2005; Zhou et al., 2004). With regard to the role of iGb3 in adaptive immune responses, Mattner et al. reported that Gram-negative, LPS-positive *Salmonella typhimurium* activates NKT cells through the recognition of iGb3, presented by LPS-activated dendritic cells. However, very recent works have cast doubt on the meaning of the iGb3 discovery (Porubsky et al., 2007; Speak et al., 2007). The study by Zhou et al. (2004) indicated that iGb3 presented by CD1d-expressing $CD4^+CD8^+$ thymocytes should be involved in the thymic positive selection of iNKT cells. Porubsky et al. has then generated iGb3 synthetase deficient mice and examined if iNKT cells are really missing in the mice lacking expression of iGb3. They found that the number and function of iNKT cells were as normal as those seen in wild-type mice. Using highly sensitive HPLC assay, Speak et al. sought for the presence of iGb3 in various mouse and human tissues. The only tissue containing iGb3 was the dorsal root ganglion of mice. No iGb3 was detected in any human tissue (Porubsky et al., 2007; Speak et al., 2007). These new findings do not support the idea that iGb3 is central in the selection of iNKT cells and re-opened the search for endogenous ligands for iNKT cells.

With regard to the pathogenesis of MS, it is interesting to know if brain-derived lipids may stimulate iNKT cells. Although such ligands have not been identified yet for iNKT cells, sulfatide derived from the myelin appears to be a ligand for non-invariant NKT cells or type II NKT cells (Godfrey et al., 2004) that bear diverse TCR repertoire although restricted by CD1d glycoprotein (Jahng et al., 2004; Zajonc et al., 2005). This interesting finding leaves room for exploring presence of myelin-derived ligands for iNKT cells that may play a role in the pathogenesis of MS.

## 5. Human iNKT cells and autoimmune diseases

iNKT cells' recognition of CD1d ligand is well known for its evolutionary conservation across species barriers as indicated by the fact that both mouse and human iNKT cells share a highly homologous CDR3 of TCR α-chain and would cross-recognize α-GalCer (Spada et al., 1998). However, iNKT cells from mouse and human significantly differ in population size in lymphoid organs and peripheral blood (mouse ≫ human). In addition, a clear functional dichotomy for $CD4^+$ and $CD4^-$

populations is found in human (Gumperz et al., 2002; Lee et al., 2002) but not in mouse (Kronenberg and Gapin, 2002). A lower number of the iNKT cells has led to repeated questions about the actual role of iNKT cells in human. However, studies have shown that human iNKT cells show an outstanding ability to proliferate after *in vitro* (van der Vliet et al., 2001a; Yanagisawa et al., 2002) or *in vivo* stimulation with α-GalCer (Chang et al., 2005). Moreover, patients with rare genetic diseases associated with the absence of iNKT cells are reported to suffer from serious viral infections (Levy et al., 2003; Rigaud et al., 2006). These results support a vital role for iNKT cells in maintaining the human health.

The $CD4^+/CD4^-$ dichotomy of human iNKT cells (Gumperz et al., 2002; Lee et al., 2002) is widely appreciated at present. In brief, $CD4^+$ iNKT cells could produce both pro- and anti-inflammatory cytokines after proper stimulation, indicating their ability to balance immune homeostasis. In contrast, $CD4^-$ iNKT cells predominantly produce proinflammatory cytokines such as TNF-α and IFN-γ, but little Th2 cytokines, which is reminiscent of NK cells rather than T cells. A number of studies have addressed the difference between $CD4^+$ and $CD4^-$ iNKT cells in human disease conditions (Araki et al., 2003; Illés et al., 2000; Takahashi et al., 2003). A striking reduction of the total number of iNKT cells in the peripheral blood from remission state MS has been reported from us in previous studies (Araki et al., 2003; Illés et al., 2000). When the $CD4^+$ and $CD4^-$ iNKT cells were analyzed separately, we again noted a remarkable reduction of $CD4^-$ iNKT cells in MS. However, a reduction of $CD4^+$ iNKT cells was only modest. Furthermore, we generated long-term $CD4^+$ iNKT cell lines from MS and healthy subjects and compared their ability to produce IFN-γ and IL-4. We found that the $CD4^+$ NKT cells from subjects with MS produce much more IL-4 than those from healthy subjects, whereas production of IFN-γ was not significantly different. The data collectively support that Th2 biased $CD4^+$ NKT cells may somehow contribute to maintaining the remission state of MS. In contrast, a Th1 bias of iNKT cells has been reported in human type I diabetes (Kent et al., 2005; Wilson et al., 1998). This bias is characterized by the inability to produce IL-4. A similar Th1 bias was also confirmed by using iNKT cell clones derived from draining lymph nodes of affected pancreas from T1D patients (Kent et al., 2005). As such, Th2 bias of iNKT cells during remission of MS seems to be purposeful, whereas the Th1 bias found in T1D could contribute to enhancing pathogenic autoimmunity.

## 6. iNKT cells regulate autoimmunity in response to exogenous ligands

By using mice lacking CD1d or TCR Jα18 gene that is required for development of iNKT cells, a number of works have proven the role of iNKT cells in self-tolerance and prevention of autoimmunity. Yet, how iNKT cells actually contribute to maintaining self-tolerance remains largely unknown. Earlier works have mainly asked how an exogenous therapeutic ligand such as OCH would modulate autoimmune disease processes. A single injection of OCH protects against development of EAE. However, a simultaneous injection of anti-IL-4 antibody abrogated the preventive effect of OCH. Moreover, disease protective effects of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from iNKT cells is involved in the disease suppression (Miyamoto et al., 2001). Thus, a single NKT cell stimulation with OCH probably inhibits EAE in an Ag-nonspecific mechanism. In contrast, it has been shown by others that repeated injections of α-GalCer would suppress T1D by promoting differentiation and recruitment of tolerogenic DCs in draining lymph nodes (Chen et al., 2005; Gillessen et al., 2003; Naumov et al., 2001). It is possible that presentation of a tissue-specific antigen by tolerogenic DCs may induce Ag-specific regulatory $CD4^+$ T cells secreting IL-10, which accounts for the protection against diabetes.

Without applying an exogenous glycolipid, Lehuen and colleagues have recently shown that iNKT cells could prevent a T cell-transfer model of diabetes by inducing an anergic state of the pathogenic, islet-specific T cells. In contrast to other related works, this suppression did not require Th2 cytokines but was dependent upon direct cell-cell contact (Beaudoin et al., 2002). Subsequent studies showed that the cellular interaction does not involve CD1d recognition by NKT cells (Kent et al., 2005; Novak et al., 2007). Although the mechanism of iNKT cell-mediated regulation in this model remains unclear, it is reminiscent of our work showing that a newly recognized NKT cells (MR1-restricted Vα19 NKT cells) would mediate immune regulation via direct contact with B cells through ICOS-ICOSL interaction independent of TCR recognition (Croxford et al., 2006).

## 7. Cytokines instruct iNKT cell response towards Th1 or Th2

Although iNKT cells could conduct a tremendous job following stimulation with exogenous ligands or via direct cellular contact, recent studies on the behavior of iNKT cells during *S. typhimurium* infection have highlighted the importance of iNKT cell recognition of an endogenous CD1d ligand in combating against microbial pathogen (Brigl et al., 2003). The work by Brigl et al. showed that iNKT cells would respond to *S. typhimurium* by producing IFN-γ, when co-cultured with DCs. Interestingly, even stimulation with LPS from *S. typhimurium* could similarly induce the IFN-γ production, indicating the involvement of TLRs rather than TCR engagement by bacterial components. Subsequent experiments showed that this IFN-γ production critically required IL-12 that was derived from DCs via TLRs in a MyD88-dependent way. However, IL-12 was not sufficient to cause the iNKT cell production of IFN-γ. It was thought that the production of IFN-γ would require recognition of endogenous CD1d ligand, as anti-CD1d antibodies proved to block the response. Whether or not iGb3 is involved is still not clear, but these results clarified that iNKT cells would exert a decisive effector function (such as a predominant IFN-γ production) when iNKT cells recognize an endogenous ligand in the presence of an exogenous cytokine.

We have recently explored if cytokines other than IL-12 may induce an effector function of iNKT cells. For this aim, human $CD4^+$ iNKT cell clones were stimulated with various cytokines in the presence of DCs. None of the clones co-cultured with

DCs exhibited any noticeable response in the absence of exogenous cytokines. However, 7 out of 27 clones examined produced a large amount of IL-5 and IL-13 when IL-2 was added to the NKT-DC co-cultures. The amount of IL-5 and IL-13 was comparable to that induced with the most potent ligand α-GalCer. However, α-GalCer never induces such a biased response but stimulates production of a broad spectrum of pro- and anti-inflammatory cytokines. Remarkable production of IL-5 and IL-13 but not of other cytokines was also confirmed by conducting DNA microarray analysis. This surprising result raises two points: 1) human $CD4^+$ iNKT cells may comprise functionally distinct populations, including such IL-5/-13 producing clones, and 2) IL-2 may be a critical factor that induces a physiological Th2 response of iNKT cells. Further analysis showed that the production of Th2 cytokines was dependent on the TCR recognition of CD1d ligand. Indeed, addition of anti-CD1d antibody blocked the response, and CD1d lacking APCs could not induce the response. Furthermore, the combination of IL-2 with a weak TCR stimulus by suboptimal concentration of anti-CD3 antibody has reproduced a similar Th2 cytokine production. These results indicate that IL-2 could play a major role in instructing the iNKT cell population to selectively produce Th2 cytokines (Sakuishi et al., 2007). Taking all these into consideration, we propose that sensing the presence of cytokines is probably one of the most fundamental abilities for the iNKT cells that are to be given only a weak TCR signal *in vivo*.

IL-12 induced production of IFN-γ (Brigl et al., 2003; Mattner et al., 2005) as well as IL-2 induced production of IL-5 (Sakuishi et al., 2007) depends upon the recognition of endogenous ligand via TCR. However, iNKT cells could also produce a large amount of cytokine in response to cytokine signals independently of TCR signals. It has been shown that iNKT cells can be activated by *Escherichia coli* LPS, and produce IFN-γ, but not IL-4. Nagarajan and Kronenberg have shown that the production of IFN-γ was dependent upon LPS-induced IL-12 and IL-18 from APC, but did not require CD1d-mediated presentation of an endogenous Ag. Furthermore, they showed that exposure to a combination of IL-12 and IL-18 sufficiently activated the iNKT cells (Nagarajan and Kronenberg, 2007). TCR-independent production of Th1 cytokine strongly indicates the innate lymphocyte-like property of iNKT cells.

## 8. Antigen presenting cells for iNKT cells

To evaluate reactivity of iNKT cells, previous works have mostly used dendritic cells (DCs) or unseparated lymphoid cells as APCs. Recently, two groups have used non-professional APCs for stimulating iNKT cells, and obtained interesting results (Bezbradica et al., 2005; Im et al., 2006). The study by Bezradica et al. has compared the ability of DCs, B cells, hepatocytes, and macrophages to present α-GalCer to mouse NKT cells. Whereas presentation with DCs induced a remarkable production of IFN-γ and IL-4 from NKT cells, α-GalCer-loaded hepatocytes or

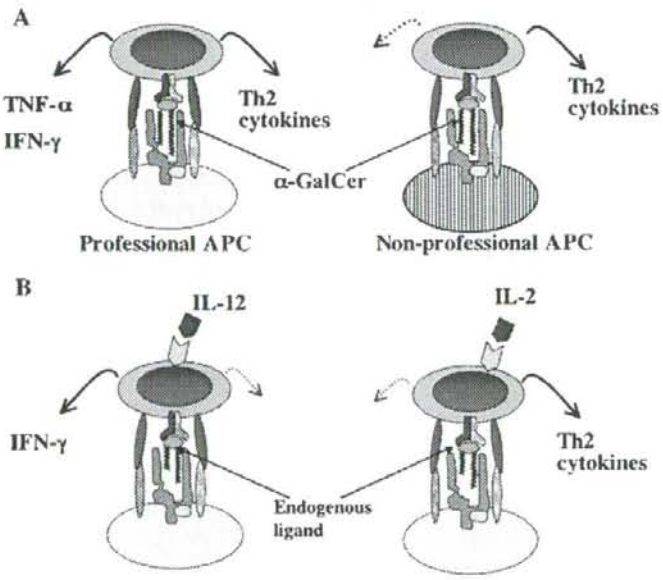


Fig. 2. Activation of iNKT cells by unconventional ways leading to functional bias. A: APC-dependent functional bias of NKT cells. Stimulating NKT cells with α-GalCer presented by professional APCs leads to production of both pro- and anti-inflammatory cytokines (left). However, when non-professional APCs such as Schwann cells (Im et al., 2006) are used, α-GalCer could induce a preferential production of Th2 cytokines from NKT cells. B: Cytokine-dependent functional bias of NKT cells recognizing endogenous ligand/CD1d. NKT cells usually exhibit only a marginal response in response to endogenous ligand bound with CD1d. However, when cytokines are added exogenously, the cells that recognize the endogenous ligand would produce a large amount of selected cytokines. For example, IL-12 induces production of IFN-γ (left) (Brigl et al., 2003), whereas IL-2 provokes IL-5 and IL-13 (right) (Sakuishi et al., 2007).

macrophages did not appear to induce iNKT cells responses. Interestingly, NKT cell stimulation with α-GalCer presented by B cells induced a weak cytokine response characterized by a low production of IL-4. Porcelli and his colleagues have examined the ability of human Schwann cells to present α-GalCer to NKT cells (Im et al., 2006). They showed that iNKT cells produced much lower amounts of proinflammatory cytokines (TNF-α and IFN-γ) but predominantly produced Th2 cytokines (IL-5 and IL-13) when Schwann cells were used as APCs. Although these studies did not examine the NKT cell reactivity to self-CD1d ligand, the results indicate that non-professional APCs tend to provoke production of Th2-associated cytokines from iNKT cells, allowing us to speculate that iNKT cell responses may greatly vary in different organs and tissues resided with different types of APCs.

## 9. Concluding remarks

Although most previous works have used α-GalCer or anti-CD3 antibody for stimulating iNKT cells to evaluate their functions, recent works have identified various alternative ways by which iNKT cells could be properly and differentially activated (Fig. 2). It is of particular note that iNKT cells exert polarized regulatory functions when exposed to an endogenous CD1d ligand in the presence of cytokines such as IL-12 and IL-2. We speculate that cytokine-triggered activation of iNKT cells should reflect a number of physiological or pathological conditions that could take place in the maintenance of immune homeostasis. Occurrence of Th1 polarization for iNKT cells or robust production of proinflammatory cytokines such as IFN-γ and osteopontin in response to infectious stimuli indicates a new mechanism for exacerbating autoimmune diseases preceded by an infection. Very interestingly, a growing number of potential agonists for iNKT cells have been identified from relatively common pathogens (Tupin et al., 2007). This opens a new possibility that environmental pathogens may play an active role in maintaining the population size and functions of iNKT cells in healthy conditions. Given that the frequency of iNKT cells in the peripheral blood greatly varies among healthy populations, this is an interesting question to be addressed experimentally. This new idea and a prevailing view about the major influence by genetic factors on iNKT cells are not mutually exclusive. Consequently, new approaches exploiting the role of iNKT cells in autoimmunity should probably consider their relation to pathogenic bacteria as well as non-pathogenic microbes.

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REVIEW ARTICLE

# Th17 Cells and Autoimmune Encephalomyelitis (EAE/MS)

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## ABSTRACT

Multiple sclerosis (MS) is a CD4+ T cell-mediated autoimmune disease affecting the central nervous system. It was largely accepted that Th1 cells driven by IL-12 were pathogenic T cells in human MS and experimental autoimmune encephalomyelitis, an animal model of MS. Recent data have established that IL-17-producing CD4+ T cells, driven by IL-23 and referred to as Th17 cells, play a pivotal role in the pathogenesis of EAE. A combination of TGF-β and IL-6 induce Th17 cell lineage commitment via expression of transcription factor RORγt. Th17 cells and induced Foxp3+ T regulatory cells are in reciprocal position in the T cell lineage commitment governed by TGF-β and IL-6. The vitamin A metabolite retinoic acid is involved in this process via TGF-β dependent induction of Foxp3. We have demonstrated that human Th17 cells could be identified as CCR2+ CCR5- memory CD4+ T cells. It is becoming clear that IL-23/Th17 axis also plays an important role in the pathogenesis of various human autoimmune diseases including MS. Additionally, accumulating evidences raise a possibility that CCR2 on Th17 cells may be a therapeutic target in MS.

## KEY WORDS

autoimmune disease, EAE, IL-17, MS, Th17 cells

## *INTRODUCTION*

Naïve CD4+ T cells begin a process of differentiation into effector T cells upon stimulation with specific antigens.[1] Th1 effector cells produce IFN-γ and TNF-α, while Th2 effector cells produce IL-4, IL-5, and IL-13.[2] Th1 differentiation requires IL-12 and the transcription factors STAT4, STAT1, and T-bet.[3,4] Th2 differentiation requires IL-4 and the transcription factors STAT6 and GATA3.[5] Th1 cells command the cellular immunity to clear intracellular pathogens, whereas Th2 cells lead the humoral immunity to control parasitic infections. However, dysregulated responses of effector T cells cause various immunopathological conditions. Namely, Th1 cells are thought to be involved in organ-specific autoimmune diseases, while Th2 cells may play important roles in allergy.

Multiple sclerosis (MS) is a chronic inflammatory disease affecting the central nervous system (CNS) white matter.[6] Activation of autoreactive CD4+ T cells specific for myelin antigens and differentiation to Th1 effectors were thought to be crucial for the development of this disease. This widely accepted theory about pathology of MS was based on data from experimental autoimmune encephalomyelitis (EAE), a rodent model of MS. However, the functional role of Th1 cells in EAE has been reconsidered upon the discovery of Th17 cells.

## *PARADIGM SHIFT FROM TH1 TO TH17*

It was previously believed that Th1 cells were pathogenic T cells in EAE because myelin-specific T cells produced large amount of IFN-γ but not IL-4 upon recall response to an immunized myelin antigen.[7] Since IL-12 was essential for the development of Th1-mediated immunity,[8] blocking IL-12 signaling was expected to ameliorate EAE. IL-12 is a heterodimeric cytokine composed of p35 and p40 subunit.[9] Using IL-12p40 and p35-deficient mice, however, it was shown that p35-deficient mice were susceptible, but p40-deficient mice were resistant to EAE.[10] The puzzle regarding pathogenesis of IL-12/Th1 response in EAE was resolved in 2003 by Cua *et al* using IL-23p19-deficient mice.[11] IL-23 is a heterodimeric cytokine that share IL-12p40 subunit with IL-12 and possess a unique p19 subunit.[9] They demonstrated that IL-23p

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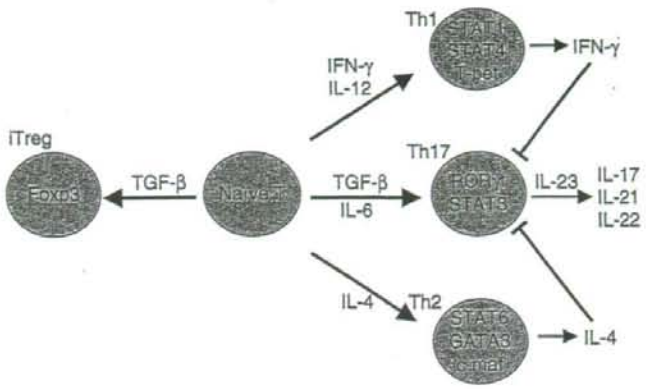


**Fig. 1** Regulation of helper T cell differentiation. Naïve CD4+ T cells differentiate into four distinct T cell subsets such as Th1, Th2, Th17 and induced T regulatory cells (iTreg) dependent on the cytokine milieu. It should be noted that the lineage commitment to either Th17 or iTreg cells is determined by IL-6 when naïve T cells are stimulated in the presence of TGF-β (reciprocal differentiation).

19 and IL-12p40, but not IL-12p35, were essential for the development of EAE.

Researching the mechanism underlying the essential role of IL-23 has revealed that autoreactive CD4+ T cells producing IL-17 were not induced in IL-23-deficient mice in EAE and collagen-induced arthritis (CIA).[12,13] IL-17 (IL-17A) is a member of IL-17 family (IL-17A-F)[14,15] and stimulates various types of cells, such as epithelial cells, endothelial cells and fibroblasts to produce proinflammatory cytokines and chemokines.[16-18] Furthermore, Th17 cells activated in the presence of IL-23 in vitro exhibited a higher capacity to transfer EAE than Th1 cells activated in the presence of IL-12.[12] These results demonstrate that IL-23/Th17 axis rather than IL-12/Th1 axis is important for the development of EAE and CIA.[19,20]

## REGULATION OF TH17 DIFFERENTIATION

Various *in vitro* differentiation systems have confirmed that IL-17 producing T cells were a distinct linage cells from Th1 or Th2 cells since differentiation of IL-17 producing T cells was promoted with blocking IFN-γ or IL-4 signaling.[21,22] Subsequently, it has been shown that a combination of transforming growth factor-β (TGF-b) and IL-6 induces differentiation of Th17 cells very efficiently (Fig. 1).[23-25] When naïve CD4+ T cells are stimulated in the presence of TGF-β, CD4+ Foxp3+ cells, but not IL-17-producing cells, are induced. Addition of TGF-β and IL-6 to naïve CD4+ T cells during the stimulation completely abrogates expression of Foxp3 and results in concomitant expression of IL-17 from these T cells, suggesting that reciprocal relationship between Th17 cells and induced T regulatory (iTreg) cells.[24] The vitamin A metabolite retinoic acid is involved in this reciprocal differentiation of iTreg and Th17 cells via TGF-β dependent induction of Foxp3.[26,27] The importance of TGF-β and IL-6 in the differentiation of Th17 cells has been further confirmed *in vivo* using IL-6 deficient mice and mice expressing a dominant negative form of the TGF-β receptor II.[28,29] Although IL-23 plays no apparent role in Th17 lineage commitment, it seems to be required for promoting survival and/or proliferation of these cells *in vivo*.[23,25] Furthermore, it has been established that IL-21, which is produced preferentially by Th17 but not Th1 cells, is important for Th17 differentiation.[29,30]

CD4+ T cell lineage commitment is regulated by specific transcription factors. Namely, Th1 differentiation requires STAT1, STAT4, and T-bet, whereas STAT6, c-maf, and GATA-3 act to promote Th2 cytokine production.[3-5] Regarding Th17 cell differentiation, retinoic acid-related orphan nuclear receptor (ROR)γt is the key transcription factor that orchestrates the differentiation of Th17 cell lineage.[31] RORγt-deficient CD4+ T cells produce no IL-17 in response to TGF-β and IL-6. Ectopic expression of RORγt induces transcription of IL-17 in naïve CD4+ T cells. STAT3, activated by IL-6 or IL-23, is also an essential transcription factor in Th17 cell differentiation via regulating RORγt.[32] In addition, Interferon regulatory factor 4 (IRF4), which has been recognized to be essential for Th2 cell differentiation, is also involved in the regulation of RORγt and differentiation of Th17 cells.[33] Among other signaling pathways IL-2 signaling via STAT5 and IL-27 signaling via STAT1 constrain Th17 cell development.[34-37]

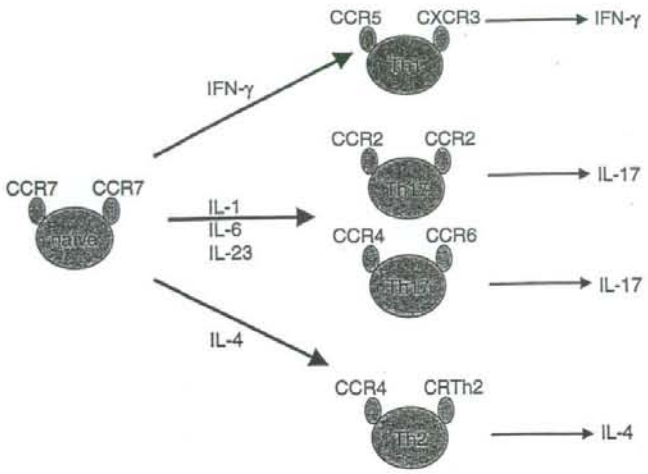


**Fig. 2** Differential expression of chemokine receptors in human helper T cell subsets. During the differentiation process $CD4^+$ T cells acquire certain sets of chemokine receptors, which confer the distinct migratory features to Th1, Th2 and Th17 cells. Others and we have identified two different Th17 populations as bearing $CCR4^+$ $CCR6^+$ and $CCR2^+$ $CCR5^-$ cells, respectively. Although the relationship between these two different populations are not fully understood, these Th17 cells may play different roles in diverse inflammatory environments.

## *HUMAN TH17 CELLS IN HEALTH AND DISEASE*

Establishment of Th17 cells as a novel Th subset in mice advances studies of human Th17 cells. Others and we have used similar methods to isolate human Th17 cells from PBMC according to expression pattern of chemokine receptors.[38,39] During the differentiation process $CD4^+$ T cells acquire certain sets of chemokine receptors, which enable the distinct positioning of Th1 and Th2 cells.[40] Namely, Th1 cells preferentially express CCR5 and CXCR3 whereas Th2 cells express CCR4, CCR8, and CRTh2.[41-43] It is conceivable that Th17 cells may also possess unique expression pattern of chemokine receptors. We have revealed that $CCR2^+$ $CCR5^-$ memory $CD4^+$ T cells produce a large amount of IL-17 and little IFN-γ, whereas $CCR2^+$ $CCR5^+$ cells reciprocally produced an enormous amount of IFN-γ but little IL-17. These results indicate that $CCR2^+$ $CCR5^-$ memory $CD4^+$ T cells belong to Th17 lineage (Fig. 2). Another group has identified another Th17 cells in PBMC as $CCR4^+$ $CCR6^+$ cells. Although the relationship between these two different populations of Th17 cells should be clarified in the future studies, these Th17 cells may play different roles in diverse inflammatory environments. The unique chemokine receptor expression pattern of Th17 cells is thought to provide a basis for their recruitment in specialized inflammatory conditions.

In vitro differentiation studies have shown that IL-1β but not TGF-β together with IL-6 or IL-23 is required for differentiation of human Th17 cells and expression of RORC, human ortholog of mouse RORγt.[44-46] These results suggest that human and mouse Th17 cells require distinct factors during differentiation.

It is becoming clear that IL-23/Th17 axis may play an important role not only in the animal models but also in human chronic inflammatory diseases. Transcripts encoding IL-17, IL-23, RORC but not IL-12 are upregulated in psoriatic lesions.[45,47] IL-22, a Th17 cell-derived cytokine, is required for IL-23-induced acanthosis, hyperplasia of the epidermis characteristic of psoriatic lesions.[48] Besides a human IL-12/23 monoclonal antibody efficiently improves psoriasis symptoms.[49] Same as EAE, IL-23/Th17 rather than IL-12/Th1 was important for animal models of the inflammatory bowel diseases (IBD).[50-53] Furthermore, in genome-wide analysis of single nucleotide polymorphisms, an uncommon coding variant of the gene encoding the IL-23 receptor conferred strong protection against Crohn's disease,[54] suggesting the IL-23 signaling pathway as a therapeutic target in IBD.

## A PATHOGENIC ROLE OF TH17 CELLS IN MS

Microarray analysis demonstrated that IL-17 transcript is upregulated in the MS lesion.[55] Concentrations of IL-17 and IL-8 in cerebrospinal fluid (CSF) are significantly higher in MS than healthy subjects.[56] The levels of IL-23 expression in monocyte-derived dendritic cells are higher in MS patients than those in healthy controls.[57] Furthermore, a recent study has shown that memory T cells producing IL-17 and IL-22 infiltrate into MS lesions.[58] These results suggest that Th17 cells may play important roles in the pathology of MS.

Although IFN-β is the most common therapy to reduce rate of relapses in MS, blockade of chemokine signaling pathways are expected to be a new therapeutic approach. Among several chemokine-chemokine receptor systems tested, CCL2 (or MCP-1)-CCR2 pathway was consistently shown to be essential for development of EAE.[59-61] Concerning MS, CCL2 is upregulated in MS lesions.[62-64] Although CCL2 levels are decreased in the CSF of MS patients,[65,66] it is explained by the mechanism that CCL2 in the CSF is consumed by the infiltrated T cells.[67] Furthermore, both IL-17 and IL-22 stimulate human Blood-Brain-Barrier endothelial cells to produce CCL2 but not CCL5 which is the ligand of CCR 5.[58] These results raise a possibility that CCL2-CCR2 signaling pathway might play an important role in migration of Th17 cells to MS lesions and that CCR2 on human Th17 cells might serve as a therapeutic target in MS.

## CONCLUSION

By discovering Th17 cells it has been revealed that these Th17 but not Th1 cells are the pathogenic T cells in EAE. Both TGF-β and IL-6 are required for differentiation of Th17 cells, while IL-23 seems to be essential for survival or expansion of this subset *in vivo*. The differentiation process is regulated by the specific transcription factor RORγt. It is necessary to reconstitute the pathological theory of MS as well as EAE from standpoint of Th17 cells. According to expression pattern of chemokine receptors, we were able to identify human Th17 cells in PBMC as $CCR2^{+}$ $CCR5^{-}$ cells. Blockade of the CCL2-CCR2 signaling pathway that guides Th17 cells to the CNS may be a therapeutic strategy in MS.

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# Treatment of neuromyelitis optica: Current debate

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***Abstract:*** Neuromyelitis optica (NMO) is an inflammatory demyelinating disease that largely affects optic nerves and spinal cord. Recent studies have identified an elevation of serum anti-aquaporin 4 antibody as a hallmark of NMO. Typical cases of NMO significantly differ from multiple sclerosis (MS) in immunological markers, histopathology, and responses to therapy. In fact, plasma exchange may be more efficacious for NMO than MS, whereas interferon-$\beta$ is recommended for MS but not for NMO. An emerging idea that pathogenesis of NMO may involve an interaction of the newly identified helper T cell subset, Th17, with B cells offers potential targets of therapy.

***Keywords:*** neuromyelitis optica, multiple sclerosis, Th17 cells, anti-aquaporin-4 antibody, interferon-$\beta$

## Introduction

Neuromyelitis optica (NMO; Devic syndrome) is an inflammatory disease of the central nervous system (CNS) that affects optic nerves and spinal cord [Jacob *et al.* 2007; Matiello *et al.* 2007; Wingerchuk *et al.* 2007]. In older literature, NMO was defined as a disorder that is characterized by development of a single episode of bilateral optic neuritis and transverse myelitis (Table 1). However, recent studies have indicated that presence of serum antibodies against aquaporin 4 (AQP4), a water channel-protein, is a hallmark of NMO and could be essential for making the diagnosis. Since anti-AQP4 antibody became recognised as a serological marker of NMO, the clinical picture of NMO has been significantly broadened. Indeed, when the latest criteria [Wingerchuk *et al.* 2006] are used for diagnosis of NMO, a large majority of the NMO patients follow a relapsing clinical course and sometimes develop brain lesions.

Of interest, NMO has been traditionally separated from multiple sclerosis (MS) in western countries, whereas they have been integrated into the category of MS in Japan, by giving a term 'opticospinal MS (OSMS)'. Because not all OSMS exhibit an elevation of anti-AQP4 antibody titer in the sera, and because OSMS may develop brain lesions characteristic of MS [Barkhof *et al.* 1997], it is still debatable as to whether OSMS and NMO may cover an entirely identical disease spectrum or not.

Nowadays, a large proportion of patients with MS are being treated with standard drugs such as interferon-$\beta$ and glatiramer acetate. It has been reported that interferon-$\beta$ may also be efficacious for NMO/OSMS based on analysis of a small number of patients [Saida *et al.* 2005]. However, more recent works have emphasized the differences in immunological and pathological features between NMO and conventional MS, which indicates the relevance of distinctive therapeutic strategies for NMO and MS. The aim of this review is to provide up-dated information on the diagnosis and treatment of NMO and also discuss the immunological pathogenesis of NMO with special reference to a critical interaction between B cells and Th17 cells, a newly identified helper T cell subset [Hsu *et al.* 2008].

## Diagnosis of NMO: discovery of anti-aquaporin 4 (AQP4) antibody and its impact

In general, the clinical picture of typical NMO is very different from that of conventional MS. Important points for differential diagnosis are as

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follows: (1) Optic neuritis in NMO could be much more serious than in MS, and often leads to blindness, (2) MRI scan of NMO often reveals presence of an extensive lesion extending over three vertebral segments (Figure 1), referred to as 'Longitudinally extensive spinal cord lesion' (LESL), (3) Oligoclonal bands (OBs) commonly found in the cerebrospinal fluid of MS is only rarely seen in NMO, (4) NMO may show brain lesions, although they are different from characteristic MS lesions. However, the patients during an early stage of NMO or those who have been actively treated may not show the characteristic clinical profile of NMO, and could be misdiagnosed. In this regard, a recent discovery of the specific serological marker of NMO (NMO-IgG or anti-AQP4 antibody) [Lennon *et al.* 2004; Lennon *et al.* 2005] has opened a new gate for diagnosis of NMO. The NMO-specific autoantibody was first identified in the sera from NMO as 'NMO-IgG' based on the ability to stain mouse CNS tissue. The target antigen of NMO-IgG was subsequently identified to be AQP4 [Lennon *et al.* 2005], which has led to establishment of assays that are more feasible and more sensitive than the original NMO-IgG assay [Paul *et al.* 2007; Tanaka *et al.* 2007; Takahashi *et al.* 2006].

Recent studies have shown that anti-AQP4 antibody or NMO-IgG can be detected in a large majority of NMO/OSMS patients, whereas most patients with conventional MS are anti-AQP4 negative [Paul *et al.* 2007; Tanaka *et al.* 2007; Nakashima *et al.* 2006]. Although, it has been argued whether NMO and MS represent distinct entities or not [Weinshenker *et al.* 2006; Kikuchi and Fukazaw, 2005], discovery of anti-AQP4 antibody has obviously strengthened the idea that typical NMO cases are distinct from MS in the pathogenesis. Furthermore, pathological analysis has recently demonstrated a remarkable loss of AQP4 [Misu *et al.* 2007; Roemer *et al.* 2007] along with concomitant absence of glial fibrillary acidic protein, a marker of astrocytes [Misu *et al.* 2007] in the lesions of NMO but not of MS. Although primary targets in MS are thought to be myelin and myelin-forming oligodendrocytes, the results of pathological studies suggest that astrocytes could be attacked by antibodies against AQP4 in NMO; further highlighting the differences between NMO and MS.

As mentioned above, patients predominantly manifesting optic nerve and spinal cord signs have been traditionally diagnosed as OSMS in Japan. A recent analysis showed that a majority of the OSMS patients are anti-AQP4 antibody positive and accompany the LESL, implying that most cases of OSMS could be diagnosed as NMO. However, some of the patients exhibited neither aniti-AQP4 nor LESL [Tanaka *et al.* 2007]. It is possible that these patients may

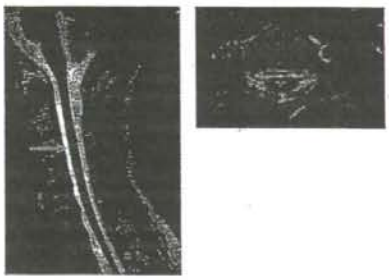
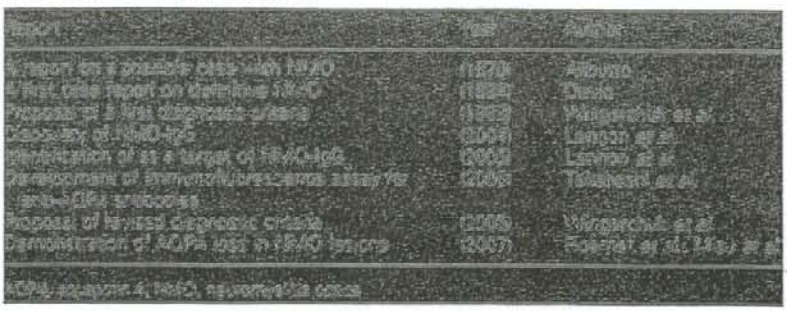


**Figure 1.** Longitudinally extensive spinal cord lesion (LESL) in a case of NMO T2-weighted cervical MRI demonstrates an extension of T2 high density involving central gray matter, which is characteristic of LESL associated with NMO.

**Table 1.** Brief history on NMO research.



belong to the category of MS, although the distribution of lesions resembles that of NMO.

Previously, presence of brain lesions and symptoms was an exclusion criterion for NMO. However, the revised diagnostic criteria allow diagnosis of NMO for patients who have brain lesions, provided that the MRI findings do not meet the diagnostic criteria for MS [Wingerchuk *et al.* 2006]. However, Matsuoka *et al.* reported on the presence of NMO patients, who have multiple juxtacortical or periventricular ovoid lesions in the brain, which is characteristic of MS, but not of NMO [Matsuoka *et al.* 2007]. Although this information may be used to argue against the distinction between MS and NMO, we would rather interpret that the patients might have both MS and NMO simultaneously. This possibility needs to be verified rigorously in future studies.

As such, discovery of anti-AQP4 antibody has greatly influenced on the understanding the pathogenesis of NMO. However, it remains unclear whether anti-AQP4 truly plays a role in the formation of destructive lesions in the optic nerve and spinal cord, although the selective loss of AQP4 in the NMO lesions indicate the pathogenic role of anti-AQP4 antibody. A number of investigators are trying to reproduce the pathology of NMO in rodents by passively transferring anti-AQP4 antibody. However, the results have not been published yet. Currently, it remains possible that pathogenic autoantibody in NMO may target CNS antigens other than AQP4.

## Cerebrospinal fluid findings in NMO

Cerebrospinal fluid (CSF) examination could also be useful for distinguishing NMO from MS. For instance, presence of prominent CSF pleocytosis ($>50 \times 10^6$ WBC/L) during acute phase could be regarded as supporting diagnosis of NMO but not of MS [Wingerchuk *et al.* 1999]. It is also of note that OBs could be detected more frequently in MS than in NMO [Bergamaschi *et al.* 2004; Misu *et al.* 2002]. Misu *et al.* previously reported that OBs are negative in the Japanese OSMS patients who have no brain lesions on MRI [Misu *et al.* 2002]. However, Bergamaschi *et al.* have recently reported that presence of OBs could be demonstrated in 27% of NMO, when CSF samples were examined repeatedly [Bergamaschi *et al.* 2004]. Notably, the authors pointed out that OBs could be continuously detected during the course of MS, whereas appearance of OBs appears to be temporary in NMO, indicating the importance of repeated CSF examination to distinguish NMO from MS. Very recently, Jarius *et al.* have reported that a polyspecific humoral response against measles, rubella, and varicella zoster virus (MRZ) was positive in 37 out of 42 CSF samples from MS, but was detected only in one out of 20 samples from NMO. They suggest that assessment of the MRZ reaction in the CSF could also help in distinguishing MS and NMO [Jarius *et al.* 2008]. Taken together, these results indicate that a combination of CSF and serum studies may further improve diagnostic certainty.

## Activation of IL-17/IL-8 axis in NMO

Besides an elevation of anti-AQP4, recent work has shown that IL-17 and IL-8 are specifically increased in the CSF from NMO [Ishizu *et al.* 2005]. IL-17 is a proinflammatory cytokine mainly produced by activated T cells, whose role in allergy and autoimmune inflammation has been highlighted lately. IL-8 is a chemokine whose major role is to recruit neutrophils. Of note, IL-8 production from macrophages and epithelial cells is promoted by IL-17. Because neutrophil infiltration is dominant in the necrotic lesions of NMO [Ishizu *et al.* 2005], the authors have argued that intrathecal activation of IL-17/IL-8 axis may uniquely contribute to the formation of destructive lesions found in NMO. If this is the case, an important question should be directed to the relationship between the IL-17/IL-8 axis and B cell immunity associated with an elevation of anti-AQP4 antibody. Though very little was known about the relationship between IL-17 and B cells, it has recently been reported that IL-17-producing T cells, namely Th17 cells [Bettelli *et al.* 2007; Steinman, 2007], would promote spontaneous formation of a germinal center and augment production of pathogenic autoantibodies in a model of systemic autoimmune disease [Hsu *et al.* 2008]. In the next section, we discuss on our hypothetical model in which the Th17 cell/B cell interaction plays a role in the pathogenesis of NMO.

### *Th17 cell biology and pathogenesis of NMO*

Th17 cells are a novel helper T cell subset distinct from Th1 or Th2. Because it has been shown that Th17 cells play a decisive role in a variety of inflammatory processes, the biology

of Th17 cells is currently the subject of broad interest [Bettelli *et al.* 2007; Steinman 2007]. Before Th17 cells were identified, studies had emphasized the role of Th1 cells that produce interferon-$\gamma$ in the pathogenesis of MS and its animal model experimental autoimmune encephalomyelitis (EAE). However, it now becomes clear that Th17 cells are crucial in the induction of EAE, and lymphocytes infiltrating the brain of MS would contain Th17 cells [Tzartos *et al.* 2008]. Although the pathogenic role of Th17 cells is sometimes being overemphasized, involvement of Th1 cells has been confirmed in various inflammatory pathologies. Interestingly, Th1 cells and Th17 cells express different sets of chemokine receptors [Sato *et al.* 2007], indicating that they might be recruited to different types of inflammatory lesions or to different anatomical sites.

Differentiation of rodent Th17 cells depends on IL-6 and transforming growth factor (TGF)-$\beta$ [Bettelli *et al.* 2007] whereas human Th17 cells appear to be induced in the presence of IL-6 and IL-1$\beta$ [Acosta-Rodriguez *et al.* 2007]. IL-23 is required for the expansion and maintenance of Th17 cells. As such IL-6 and IL-23 are now thought to be key cytokines in the generation of pathogenic Th17 cells.

The relation between Th17 cells and production of anti-AQP4 antibody is still not clear but could be speculated on the results of animal experiments. It is noteworthy that IL-17 produced by Th17 cells has recently been found to promote the germinal center formation in a spontaneous autoimmune disease model by altering the B cell chemotactic response, which leads to a massive production of pathogenic autoantibody [Hsu *et al.* 2008]. In contrast, blocking IL-17 signaling was inhibitory to the production of autoantibody and prevented the development of the autoimmune disease. These results indicate that Th17 cells would contribute to augmenting B cell autoimmunity through a mechanism distinct from its proinflammatory action. Notably, presence of a germinal center-like structure was demonstrated in the subarachnoid space of a rodent NMO model, which has been created by introducing genes for both T cell receptor (TCR) and B cell receptor for myelin oligodendrocytes glycoprotein (MOG) [Bettelli *et al.* 2006; Krishnamoorthy *et al.* 2006]. The mice spontaneously develop optic neuritis and myelitis. Furthermore, it is thought that collaboration of T cells (Th17) and B cells play a critical role in shaping the unique lesion distribution in this mouse model. If human NMO also involves a Th17 cell/B cell interaction, cytokines, chemokines and their receptors that play a role in Th17 cell-dependent production of pathogenic autoantibody could be potential therapeutic targets in NMO. The hypothetical model will be verified in a future study.

## Interferon-$\beta$ and NMO

Although a small preliminary report suggests the efficacy of interferon-$\beta$ on OSMS [Saida *et al.* 2005], another study does not recommend its use for NMO in comparison with immunosuppressive agents [Papeix *et al.* 2007]. The most prominent and common side effects of interferon are a flu-like syndrome of fever, headache, myalgia, arthralgia, and general malaise. Furthermore, there are several case reports in Japan documenting a worsening of NMO [Warabi *et al.* 2007] or development of large brain lesions in NMO patients after starting interferon-$\beta$ [Shimizu *et al.* 2008].

Although the clinical reports need to be carefully analyzed before making a conclusion, some cautions should be made upon the fact that type I interferon (including interferon-$\alpha$ and $-\beta$) would worsen or trigger the development of some antibody-mediated autoimmune diseases. For example, therapeutic use of type I interferon for cancer and hepatitis has been shown to cause exacerbation of SLE, thyroiditis, diabetes, psoriasis, rheumatoid arthritis, autoimmune hemolytic anemia, and myasthenia gravis [Baccala, *et al.* 2005; Theofilopoulos *et al.* 2005; Gota and Calabrise 2003; Stewart, 2003]. Among these, SLE and type I interferon has been causally linked following intensive analysis [Banchereau and Pascual, 2006; Pascual *et al.* 2006]. Early studies reported increased serum levels of IFN-$\alpha$ in lupus patients, which correlate with disease activity [Kim *et al.* 1987l; Ytterberg and Schnitzer, 1982]. More recently, microarray studies have identified increased expression of interferon-$\alpha$- and interferon-$\gamma$-induced genes in peripheral blood lymphocytes of SLE patients in correlation with disease severity [Bennett *et al.* 2003; Baechler *et al.* 2003; Crow *et al.* 2003; Han *et al.* 2003]. Consistently, interferon-$\alpha$ was recently identified as the serum factor in SLE that could induce differentiation of dendritic cells with efficacious

antigen-presenting ability [Blanco *et al.* 2001]. Type I interferon might also contribute to immune complex formation in SLE by directly activating B cells [Le bon *et al.* 2001]. These results highlight the augmenting effect of type I interferon on antibody-mediated autoimmunity, which differs greatly from that of MS.

It is also of note that interferon-$\beta$ shows a potential to induce IL-6 *in vivo* [Satoh *et al.* 2006] and *in vivo* [Nakatsuji *et al.* 2006]. IL-6 is a key cytokine involved in the induction of Th17 cells as well as growth and differentiation of B cells. Satoh *et al.* examined the gene expression profile of peripheral blood lymphocytes after culture with interferon-$\beta$ and found a number of inflammatory cytokines including IL-6 are upregulated. Nakatsuji *et al.* has shown that the level of serum IL-6 after injection of interferon-$\beta$ would correlate with side effects such as headache in the patients with MS, but ironically also predict the efficacy of interferon-$\beta$ treatment in MS. Taken these together, injection of interferon-$\beta$ could lead to induction of IL-6 at least transiently. From a theoretical point of view, one may argue that the IL-6-stimulatory property of interferon-$\beta$ is not beneficial for treating NMO involving B cells and Th17 cells, both of which are responsive to IL-6. A systematic retrospective survey for interferon-$\beta$ treated NMO patients will clarify if this concern is appropriate or not.

According to recent studies, abnormalities found in the brain MRI of NMO ranged from 10 to 50%. Asymptomatic brain lesions are now thought to be common in NMO, and symptomatic brain lesions do not exclude the diagnosis of NMO. Cabrera-Gómez *et al.* has reported that none of the brain MRI abnormalities in NMO were compatible with the criteria of MS brain lesions proposed by Barkhof *et al.* (1997) [Cabrera-Gómez *et al.* 2007]. As an extreme example, we show a patient with NMO, who developed a few large lesions in the brain white matter two months after starting interferon-$\beta$ (Figure 2). A recent report by Shimizu *et al.* has also described the presence of similar NMO patients who developed large brain lesions after starting interferon-$\beta$ [Shimizu *et al.* 2008]. The initial clinical and radiological features of our patient were consistent with NMO, and anti-AQP4 antibody was positive. This case suggests to us that a unique pattern of NMO lesion distribution could be transformed into another pattern of disease after undergoing

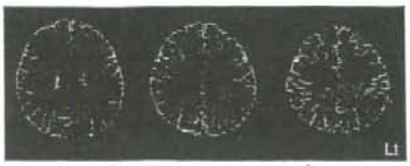


**Figure 2.** Development of large white matter lesions in a case of neuromyelitis optica (NMO) 2 months after starting interferon-$\beta$ This young female patient was aquaporin 4 antibody-positive and showed a clinical and radiological picture characteristic of NMO. However, two months after starting interferon-$\beta$ 1b treatment, she developed signs of brain hemispheres and MRI showed multiple large white matter lesions.

imunomodulation. We also speculate that interferon-$\beta$ treatment might have triggered the unusual relapse in NMO.

### Therapy of NMO in practice

At present, very little information is available that helps physicians and patients choose the best treatment for NMO. In general, treatment of acute exacerbation of NMO may start with intravenous corticosteroids (typically 1,000 mg of methylprednisolone for 3–5 consecutive days). Because the efficacy of plasma exchange was reported in NMO-IgG-positive patients with NMO [Watanabe *et al.* 2007a], plasmapheresis could be considered if clinical improvement is not satisfactory. However, effects of plasmapheresis are not consistent, and anti-AQP4 antibody could rise rapidly after plasmapheresis (Figure 3). To prevent the rebound of pathogenic antibody titers after plasma exchange, a combination therapy with immunosuppressive agents may be needed in some cases. Figure 3 demonstrates the clinical course of representative patients who were treated with plasmapheresis (plasma exchange or immunoadsorption (IA)). In the first case (Figure 3(a)), intravenous methylprednisolone (IVMP) treatment was found to reduce anti-AQP4 antibody titers in the serum, which was accompanied with some clinical improvement. However, as residual symptoms were not tolerable, plasma exchange was subsequently applied, which led to further recovery and disappearance of anti-AQP4 antibody. In the second case (Figure 3(b)), IVMP treatment was followed by plasmapheresis by using IA. We found that the first course of the IVMP plus IA tended to increase the titers of

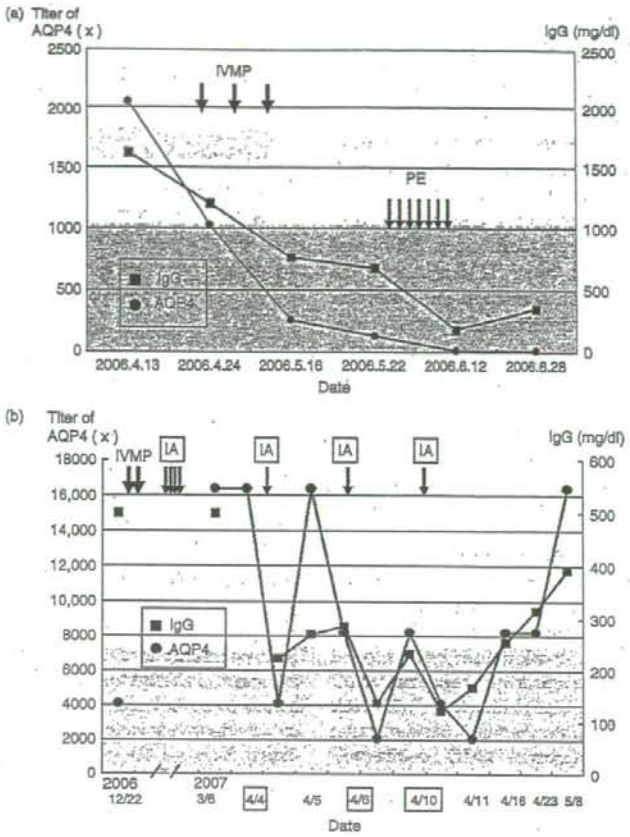



**Figure 3.** Treatment of NMO with plasmapheresis: representative cases (a) This 36-year-old female developed dysesthesia of the right leg and a constrictive band sensation in the chest region. A few days later, she experienced high fever, the loss of visual perception, progressive muscle weakness, and severe disturbance of sensation in all the limbs. She could not stand and suffered from neurogenic bladder. Treatment was initiated by the administration of 1000 mg/day of methylprednisolone (IVMP) for three consecutive days; this was followed by plasma exchange (PE) therapy which was conducted seven times over a two-week-period. The treatment was judged successful by clinical improvement as well as reduction of anti-aquaporin 4 (AQP4) antibody. (b) This 54-year-old female became completely paraplegic and was confined to bed after the development of thoracic transverse myelitis in December 2006. Although IVMP (1000 mg/day for five days followed by 500 mg/day for three days) and immunoadsorption (IA) therapies (four times) were applied, anti-AQP4 titers were somewhat elevated. So we checked the anti-AQP4 titer and total IgG before and after each of successive IA sessions. IA effectively removed the antibody and reduced the IgG amount after every IA session. But the titer and IgG returned rapidly. The anti-AQP4 antibody exhibits a higher rate of return to the basal level than that of the serum IgG. On evaluation on one month after the last IA, the patient's clinical improvement was very limited, and the anti-AQP4 antibody titer returned to the level of before starting the treatment.

anti-AQP4 antibody eleven weeks after starting the treatment. Subsequently, we measured the antibody titers and amount of serum IgG before and after each successive IA treatment. On each occasion, IA effectively removed the antibody and reduced the IgG amount. However, anti-AQP4 as well as total immunoglobulins recovered very quickly and returned to the pre-treatment level one month after the last IA. We attempted to add an immunosuppressive

drug, but the patient could not tolerate the side effects. The unsatisfactory result indicates that the primary target of therapy should be plasma cells producing pathogenic autoantibody.

To control the production of antibody, azathioprine could be used during the remission phase of NMO, often in combination with oral prednisone. Mandler *et al.* treated seven patients with newly diagnosed NMO with prednisone and azathioprine for 18 months. They found that relapses were prevented completely for more than 18 months and the patients improved significantly in the Expanded Disability Status Scale score [Mandler *et al.* 1998]. Figure 4 shows the clinical course of an anti-AQP4 antibody positive NMO patient being treated in our clinic. This NMO patient was in a state of remission for almost four years after two clinical attacks. However, she suddenly developed optic neuritis and myelitis at 57 years of age, and then interferon-$\beta$ 1b therapy was introduced. The patient did not respond to the therapy, and clinical activity seemed to be even exacerbated. Because of frequent relapses, azathioprine (100 mg/day) was prescribed in addition. The patient then entered a state of remission, which was maintained even after stopping interferon-$\beta$. This interesting case indicates the efficacy of azathioprine in NMO.

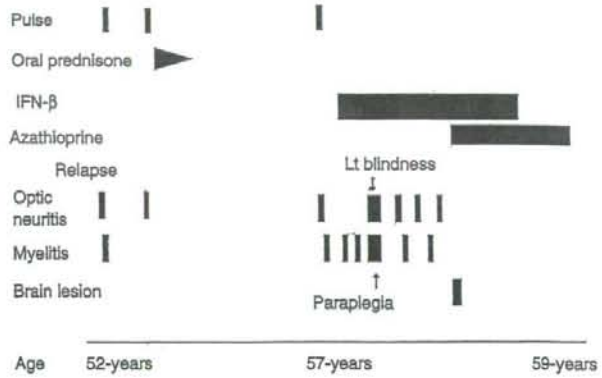



**Figure 4.** A patient with NMO who did not respond to interferon-$\beta$ (IFN-$\beta$) but to azathioprine. Interferon-$\beta$ was introduced to this female patient with NMO, as the patient's condition became active. However, there was no noticeable clinical benefit. After adding azathioprine, the patient entered a good remission state without any signs of relapses. Subsequently, we have withdrawn interferon-$\beta$, and the remission state is still continuing.

Recently, a retrospective investigation revealed that low-dose corticosteroids might reduce the rate of relapses in NMO [Watanabe *et al.* 2007b]. In some NMO patients, monthly intravenous infusion of immunoglobulin was reported to be effective [Bakker and Metz 2004]. Intravenous infusions of mitoxantrone hydrochloride (12 mg/m$^2$, monthly for six months followed by three additional treatments every three months) appeared to reduce relapses [Weinstock-Guttman *et al.* 2006]. As mitoxantrone would very potently suppress B-cell immunity directly or through a macrophage-mediated mechanism [Fidler *et al.* 1986], its efficacy in NMO is not unexpected. An open-label study of rituximab (a monoclonal antibody specific for CD20$^+$ B cells) showed an effective outcome for NMO [Cree *et al.* 2005]. Rituximab is an attractive treatment option for NMO because of its selective action against B cells. However, the potential risk and side effects should be taken into consideration. As an alternative therapeutic option, a single case report showed the efficacy of mycophenolate mofetil (2 g/day), which controls T cell-dependent antibody responses through purine synthesis inhibition [Falcini *et al.* 2006]. There is also a case report suggesting efficacy of glatiramer acetate on NMO [Bergamaschi *et al.* 2003].

## Concluding remark

NMO is an autoimmune CNS disease characterized by the presence of anti-AQP4 antibody. According to the latest criteria for diagnosis, typical cases of NMO could be easily differentiated from MS by measuring anti-AQP4 antibody and examining the presence of LESL by spinal MRI. However, patients who have been treated with interferon-$\beta$ or immunosuppressive drugs may show an atypical presentation, such as association of large brain lesions or clinical presentation of NMO without accompanying detectable anti-AQP4 antibody titers. Moreover, if the available anti-AQP4 assay is not sensitive enough, it might be hard to make a conclusive diagnosis of NMO. Interestingly, transgenic mice bearing MOG-specific T cell and B cell receptor are reported to exhibit NMO-like pathology, in which collaboration between T cells and B cells is critical [Bettelli *et al.* 2006; Krishnamoorthy *et al.* 2006]. By contrast, it remains unclear whether anti-AQP4 antibody may be truly pathogenic. It is rather promising to target B cells by a