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Eomes-expressing T-helper cells as potential target of therapy in chronic neuroinflammation

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ABSTRACT

Reserch progresses in understanding the pathogenicity of multiple sclerosis (MS) in the last couple of decade has enabled us to develop new drug entities available in the clinic. However, we still have not succeeded in preventing conversion from relapsing-remitting MS (RR-MS) to secondary progressive MS (SP-MS) and curing this intractable form of MS. Furthermore, diagnosis is usually retrospective and subjective, relying on gradual worsening of neurological signs/symptoms. This is obviously due to the lack of understanding for the pathogenicity driving disease progression in MS and of reliable biomarkers reflecting the progressive or stationary disease status. Two relevant components are involved in brain pathology of SP-MS, neurodegeneration and inflammation. Neurodegeneration may occur spontaneously in a neuron-intrinsic manner under chronic inflammation, such as glutamate excitotoxicity, mitochondrial/oxidative injury with iron deposit in the brain, and loss of trophic support. Meanwhile, inflammation is usually associated with recurrent relapse and the cumulative infiltration of immune cells, including T cells, B cells, and myeloid cells of peripheral or CNS origin, could ignite the processes of neurodegeneration. Especially, the higher frequency of leptomeningeal follicle-like structures observed in SP-MS patients suggests that immune cells sheltered behind a blood-brain barrier is still active under smoldering CNS inflammation. Recent successes in Ocrelizumab for primary progressive in MS (PP-MS) and Siponimod for SP-MS reappraised the importance of immune cells for pathogenesis progressive MS. Accordingly, our recent comparative analysis between MS and its animal model, experimental autoimmune encephalomyelitis (EAE), raises a new possibility that ectopic expression of eomesodermin (Eomes) in helper T (Th) cells constitutes a previously unappreciated subset of Th cells with cytotoxic potential against neuronal cells. In this review article, I will summarize the mechanisms proposed on pathogenesis of SP-MS and propose a new pathogenic mechanism for neurodegeneration mediated by unique cytotoxic Th cells.

1. Introduction

Due to the rapid and spectacular technological innovation in genetic analysis, genome-wide association studies have successfully revealed the involvement of a significant number of genes associated with the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) (Genome-wide association s, 2007; Rioux et al., 2007; Baranzini and Oksenberg, 2017; Sawcer et al., 2011). Interestingly, most of the susceptibility genes associated with the pathogenesis of MS have been integrated into those involved in the regulation of helper T (Th) cell function and cellular immune responses (Sawcer et al., 2011). These data clearly demonstrate the pivotal roles of acquired immunity, especially regulation and maintenance of differentiation and functions of Th cells, on the development of relapsing-remitting MS (RR-MS). During the disease course of MS, an inflammatory cascade triggered by infiltration of pathogenic T cells that secrete interferon (IFN)-γ, interleukin (IL)-17, and other inflammatory cytokines promotes demyelination and axonal degeneration characteristic of MS lesions (Gold et al., 2006; Man et al., 2007; Pierson et al., 2012). Consequently, better understanding of pathogenesis of the disease during past decades has enabled the expansion of disease-modifying therapies. Namely, conventional IFN-β that reduces relapse rates and severity through modulation of general immune responses has long been used for treatment of MS. Meanwhile, anti-α4 integrin antibody (Natarizumab) and sphingosine-1-phosphate receptor type 1 (S1P1R) agonist (fingolimod) that inhibit the mobilization and accumulation of T cells into central nervous system (CNS), and glatiramer acetate (Copolymer-1) that attenuates excessive T cell activation through acting as a decoy for myelin antigens are introduced recently.

In contrast, current therapeutic options for progressive forms of MS,

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including primary progressive MS (PP-MS) and secondary progressive MS (SP-MS), remain poor in comparison with those for RR-MS. Furthermore, diagnosis for SP-MS is based on patients' medical history of neurological deterioration with or without relapses after long-term medical follow-up. Although classification criteria for progressive MS have been recently revised (Lublin et al., 2014), the diagnosis of SP-MS remains retrospective in principle. Here, precise diagnosis of progressive MS requires novel biomarkers that faithfully reflect ongoing progression of the symptoms in SP-MS. One apparent reason for this is a lack of precise understanding for pathomechanisms driving the progressive form of MS of and reliable biomarkers reflecting their disease status, which enable identification of therapeutic target molecule(s) and differential diagnosis (Correale et al., 2017). RR-MS and SP-MS are demonstrated to have different pathological features. RR-MS is more associated with massive inflammation, recurrent entry of immune cells into CNS, disturbance of blood-brain barrier (BBB), and active CNS lesions. Meanwhile, SP-MS is more relevant to smoldering inflammation restricted within CNS, leptmeningeal inflammatory aggregates (follicle-like structure), slow expansion of persistent lesions, subpial cortical demyelination, and brain atrophy. There are numerous hypothetical mechanisms that explain leptmeningeal formation of follicle-like structure, cortical demyelination, and neurodegeneration associated to SP-MS. Neurodegeneration and chronic inflammation are considered to be two components involved in brain damage in SP-MS. However, development of neurodegeneration and chronic inflammation could occur either simultaneously or independently and mutual association between these two components is not clear so far. In this review, I will summarize the possible mechanisms proposed to date on pathogenesis of SP-MS. I would also propose a new pathogenic mechanism for neurodegeneration by unique cytotoxic Th cells based on our recent progress in research for SP-MS pathogenesis (Raveney et al., 2015).

2. Currently accepted pathogenic mechanism of secondary progressive MS

2.1. Neuron-intrinsic mechanisms of neurodegeneration and axonal dysfunction

Prevention of progression in neurological diseases such as MS and many other neurodegenerative diseases remain an obvious challenge in the field of clinical research. Although it is not well elucidated why and how conversion of the disease from RR-MS to SP-MS takes place, inflammation and neurodegeneration, which are not necessarily mutually exclusive, are two key components strongly associated with progressive MS (Lassmann et al., 2012; Larochelle et al., 2016; Lassmann, 2010). Currently, CNS-autonomous events associated with neurodegeneration such as microglial activation, mitochondrial injury, glutamate excitotoxicty, loss of trophic support etc. are believed to be major components associated with progressive MS.

2.1.1. Microglial activation

Microglial activation is one of the most common features of active tissue injury not only in MS, but also in other neuroinflammatory and neurodegenerative diseases (Lassmann, 2014). Interestingly, activation of microglia is not restricted to lesions, but is observed in normal-appearing white and grey matter of patients with progressive MS (Kutzelnigg et al., 2005). Therefore, microglial activation is prerequisite for foundation of prolonged inflammatory milieu in the CNS, followed by active lesion formation and additional components are required to trigger variable tissue damage. Activated microglia are proposed to damage directly both oligodendrocytes and neurons via secretion of pro-inflammatory cytokines and reactive oxygen and nitrogen species (ROS/RNS), respectively (Correale, 2014; Nikic et al., 2011).

2.1.2. Mitochondrial injury

Mitochondria are known to be highly susceptible to oxidative injury and ROS/RNS impair activity of respiratory chain complexes in mitochondria. Therefore, consequent energy failure caused by mitochondrial dysfunction can lead to axonal degeneration and neuronal cell death (Campbell et al., 2012). Furthermore, mitochondrial damage may induce release of oxygen radicals as a byproduct of impaired respiratory chain reaction, forming a vicious cycle of tissue injury (Murphy, 2009). Interestingly, iron released from dying oligodendrocytes accumulates in microglia, astrocytes, and neurons at edges and centers of lesions in MS patients (Hametner et al., 2013).

2.1.3. Glutamate excitotoxicty

Glutamate is one of the major excitatory neurotransmitter of the CNS. Functional dysregulation of glutamate transporter leads to an abnormal increase of extracellular glutamate. Excitotoxicity caused by exposure to excess glutamate is directly toxic to neurons (Matute et al., 1997). Indeed, sustained activation of glutamate receptors induces an excess of intra-axonal Ca2+ and concomitant axonal degeneration. However, the cellular source of glutamate involved in individual processes of RR- and progressive MS could differ and it is still controversial whether normalization of excess glutamate release is feasible or not. In any case, glutamate-scavenging therapy employing glutamate oxaloacetate transaminase (GOT) may be beneficial for treatment of MS (Honorat et al., 2017).

2.1.4. Dysregulated distribution of ion channel

Energy failure and demyelination induce unapt function and distribution of ion channels, which are commonly observed during the course of neurodegeneraion as imbalanced axonal ion homeostasis. Following demyelination, Na^+ channels, voltage-gated Ca^{2+} channels, acid-sensing ion channels and transient potential receptors are dispersed diffusely along the bare axonlemma after demyelination, eventually leading to axonal Ca^{2+} overload (Black et al., 2007; Friese et al., 2014; Vergo et al., 2011). On the other hand, modification of the dysregulated distribution of ion channels may prevent neurodegeneration and therefore provide a novel neuroprotective intervention for MS therapies (Waxman, 2006).

2.2. Immune-mediated neuronal damage associated with neurodegeneration

The fact that global inflammatory responses in the CNS of SP-MS patients are much less apparent compared with acute disease makes us underscore the significance of underlying smoldering inflammation for the development and progression of SP-MS. Although sustained low-grade inflammation is attributed to intermittent stimulation of innate immunity, including activation of microglia and dendritic cells in the CNS (Karni et al., 2006; Weiner, 2009), the precise mechanism of this activation remains elusive. SP-MS by definition is a progressive form of disease preceded by repetition of relapse/remission with massive inflammation through activation of acquired immunity. Although numbers of infiltrated immune cells at the progressive stage of MS are relatively low, it doesn't necessarily exclude the possibility that those immune cells are involved in the pathogenesis of SP-MS. Actually, Siponimod (BAF312), a novel selective sphingosine-1-phosphate receptor modulator, has been demonstrated to delay disability progression in patients with SP-MS in a recent clinical trial (Gajofatto, 2017) and ocrelizumab, a humanized anti-CD20 monoclonal antibody, was shown to reach the primary disability endpoint against PP-MS patients (Gajofatto et al., 2017), suggesting the possible involvement of immune components such as T cells and B cells in the pathogenesis of inflammatory CNS diseases with neurodegenerative symptoms. There are a number of evidences that implies the involvement of immune components as discussed below.

2.2.1. CNS T cells

The degree of inflammation observed in RR-MS is gradually less apparent in SP-MS in parallel to increased age and disease duration. Peripheral immune activation targeting the CNS causes MS pathology during the early phases of RR-MS. In contrast, inflammatory immune responses in SP-MS are more compartmentalized within CNS due to recovery of damaged blood-brain barrier. Therefore, it is difficult to monitor ongoing immune responses within the CNS in SP-MS. In addition, potential association between Epstein-Barr virus (EBV) infection and the development of MS is well described. EBV antigens derived from lytic cycle proteins trigger activation of $CD8^+$ T cells (Hislop et al., 2007), which induce accumulation of $CD8^+$ T cells into cortical CNS lesions with MS. In vitro analysis revealed that $CD8^+$ T cells are shown to kill neurons through the Fas/FasL-dependent pathway in MHC class I-restricted manner (Medana et al., 2000; Giuliani et al., 2003). Furthermore, cytotoxic T cells release pro-inflammatory cytokines such as IFN-γ, TNF-α and cytolytic molecules (granzyme, perforin, and granulysin) (Huse et al., 2008; Meuth et al., 2009). IFN-γ induces glutamate neurotoxicity through IFN-γ/AMPA GluR1 receptor complex (Mizuno et al., 2008). TNF-α enhances neuronal cell death via TNF receptor p55 and the combination of granzyme and perforin harms neurons (Venters et al., 2000). Although cytotoxic T cells may be involved in pathogenesis of SP-MS, the underlying mode of action how they damage neurons in MS is still controversial. Pathogenic involvement of $CD4^+$ T cells in SP-MS is much less clear, but the above-mentioned microglial activation may actively present antigens and stimulate $CD4^+$ T cells infiltrated into CNS (Correale, 2014). In a number of in vitro studies, TRAIL ligand secreted by $CD4^+$ and $CD8^+$ cells induces apoptosis of neurons (Aktas et al., 2005; Nitsch et al., 2000; Vogt et al., 2009). Perforin and granzyme A/B secreted by $CD8^+$ (and to a lower degree by $CD4^+$ cells) may induce antigen-dependent neuronal apoptosis (Meuth et al., 2009). Fas induces apoptosis of neurons after binding to Fas ligand expressed on $CD8^+$ T cells (Medana et al., 2000). In addition T cell-derived inflammatory cytokines sensitize glutamate receptors and increase glutamate excitotoxicity. Namely, TNF-α augments AMPA-induced toxicity in Purkinje neurons by increasing intracellular calcium flux (Bliss et al., 2011) and IFN-γ modulates AMPA receptor clustering and synaptic activity in hippocampal cell culture (Vikman et al., 2001). Furthermore, IL-17 promotes NMDA NR1 phosphorylation and induces thermal hyperalgesia (Meng et al., 2013). Recently, we have identified another subset of $CD4^+$ T cells constitutively expressing eomesodermin (described as Eomes hereafter) that exert unexpected neurotoxic properties and may be relevant to the pathogenesis of SP-MS as described below (Raveney et al., 2015).

2.2.2. CNS B cells

The function of B cells potentially relevant to the pathogenesis of SP-MS is the active production of disease-promoting antibodies, secretion of pro-inflammatory cytokines that may modify the function of other immune cells, and antigen presentation through cognate interaction with corresponding T cells. Abnormal B cell responses for cytokine production are suggested to trigger T cell-mediated disease progression in MS (Bar-Or et al., 2010). Interestingly, comparative analysis of the B cell repertoire derived from brain tissue of progressive MS patients revealed that the majority of B cell clones use IgG isotype and show shared repertoire distribution between meningeal follicles and parenchymal infiltrates (Lovato et al., 2011). Another report demonstrated that infiltration of plasma cells into the CNS is more frequent in SP-MS than RR-MS (Frischer et al., 2009).

2.2.3. Ectopic formation of follicle like structures (FLS) and concealed immune responses behind BBB

Ectopic formation of FLSs have been found in 40–70% of SP-MS patients especially in the subarachnoid space of leptomeninges, close to inflamed blood vessels, but not in PP-MS patients by postmortem brain dissection (Magliozzi et al., 2007; Howell et al., 2015; Serafini et al., 2004, 2016). Developmental stages of FLS are diverse from newly-formed and immature cellular aggregates, to highly-organized cellular mass resembling tertiary lymphoid structures. FLS are composed of a variety of immune cells including T lymphocytes, B lymphocytes, and plasma cells with co-existing follicular dendritic cells. Follicular dendritic cells secrete CXCL13 and induce recruitment and maturation of B cells (Corsiero et al., 2012). Therefore, the activation of acquired immunity in this special compartment may contribute to cortical pathology in SP-MS (Lassmann et al., 2007; Magliozzi et al., 2010). This is of particular relevance to the progressive form of MS where the integrity of the BBB is recovered, making CNS an isolated compartment, independent of peripheral immunological properties. Interestingly, cortical demyelination, neurodegeneration and brain atrophy demonstrate positive correlation with diffuse inflammatory infiltrates of immune cells and formation of FLSs in leptomeninges (Magliozzi et al., 2007, 2010; Lassmann et al., 2007), suggesting that immune activation in the CNS contributes to cortical pathology in progressive MS. Of note, a clear gradient of neuronal loss is observed in grey matter lesions and normal-appearing grey matter in the motor cortex of follicle-positive SPMS cases. This is greater in superficial cortical layers nearer the pial surface than in inner cortical layers accompanied by glia limitans damage with astrocyte loss. Interestingly, density of activated microglia shows an opposite gradient with numbers of neurons in both grey matter lesions and normal-appearing white matter only in follicle-positive SPMS cases. These data imply that cytotoxic factors diffusing from the meningeal compartment may play an essential role in the development of subpial cortical lesions and the consequent increase in clinical disability (Magliozzi et al., 2010). Nevertheless, some follow up studies have not successfully observed substantial perivascular infiltration in intracortical lesions in postmortem brains from patients with longstanding progressive multiple sclerosis. Considering that the development of SP-MS is preceded by a number of relapses and remissions concomitant with inflammatory immune responses during the course of RR-MS, these data may suggest that formation of FLSs requires repeated inflammatory responses in the CNS and may be primarily formed during the relapsing remitting phase of the disease. In other words, formation of FLSs may be unstable, relatively short-lived, and intermittently structured after the development of active white matter lesions (Amit Bar-Or, Special symposium at the Japanese Society of Neuroimmunoly meeting 2017). Recent progresses in clinical trials of Ocrelizumab for PP-MS (Gajofatto, 2017) and Siponimod for SP-MS (Gajofatto et al., 2017) strongly implicate the involvement of immune components of acquired immunity such as T cells and B cells.

2.3. Aging

It is quite important to point out that above mentioned chronic low-grade immune activation is one of the typical features of aging (inflamm-aging) and of chronic viral infection such as cytomegalovirus (CMV), human immunodeficiency virus 1 (HIV-1), human hepatitis virus B and so on. (Gemechu and Bentivoglio, 2012; Kared et al., 2014; Broux et al., 2012a; Thewissen et al., 2005). Interestingly, repeated exposure to ordinary protein antigens induces so-called immunosenescence, which is a shared immunological status in patients with chronic inflammatory diseases such as MS. For example, expansion of $CD4^+$CD28neg effector memory T cells is commonly observed with aging, as well as in some autoimmune disease patients including MS (Broux et al., 2012a; Markovic-Plese et al., 2001; Miyazaki et al., 2008; Scholz et al., 1998). Typical features of $CD4^+$CD28neg T cells include resistance to apoptosis and refractory to regulatory T cell-mediated suppression (Kared et al., 2014; Markovic-Plese et al., 2001; Thewissen et al., 2007). In addition, these $CD4^+$CD28neg T cells usually express higher levels of adhesion molecules and acquire cytolytic properties (Scholz et al., 1998; Thewissen et al., 2007). CX3CR1, fractalkine receptor, is exclusively expressed in $CD4^+$CD28neg T cells and drive their recruitment into CNS of MS patients (Broux et al., 2012b). Aging also

affects the phenotype of conventional T cell subsets. For instance, accumulation of functionally impaired Th17 cells and Treg cells are apparent and $CD8^+$ T cells acquire increased cytotoxic property upon aging. Furthermore, diversity of B cell repertoire decreases and conversely cross-reactivity to self-antigens increases (Scholz et al., 2013). Infiltration of plasma cell into CNS is more frequent in SP-MS than RR-MS, possibly due to dominant expansion of plasma cells associated with aging (Frischer et al., 2009). Taken together, senescent immune cells with senescent properties might not be as functional as normal immune cells with adolescent properties and the previously unappreciated influence of aging on immune cell functions and their responses to disease-modifying therapy must be re-considered in the future in order to unveil the enigmatic pathogenesis of autoimmune diseases with chronic low-grade immune activation such as SP-MS.

3. Therapeutic consideration for treatment of SP-MS and the accumulated outcome of past clinical trials

In general, MS pathology is composed of three mutually-relevant stages based on its pathological features (Lassmann, 2017), a first stage of brain injury with massive demyelination due to autoimmune CNS inflammation, a second stage of refractory inflammation segregated within the CNS compartment, and a third stage of progressive neurodegeneration with smoldering neuroinflammation. Above-mentioned CNS events associated with pathogenesis of progressive MS emerge either independently or in mutually-entangled manner during the latter two stages of neurodegeneration. However, because scientific and medical substantiation for molecular and cellular mechanisms of SP-MS is inadequate and inaccurate to date, the development of therapeutic candidates has not been necessarily successful. One apparent reason is that most clinical trails testing compounds for progressive MS have based on the common study designs and endpoints applied for RRMS, which may not be suitable and sensitive enough to grab the particular properties of progressive diseases, making it difficult to evaluate drug efficacy (Lublin et al., 2016). Actually, most drugs tested in progressive MS trials were not specifically designed to intervene progressive form of MS, but were on an extension of clinical trials in RRMS. In spite of the long history of struggle with a high failure rate of clinical trials for progressive MS, there are a number of trials that ended up in success as summarized below (Tur and Montalban, 2017).

Although IFN-1b is the first drug that achieved a primary endpoint of beneficial effect in delaying disability progression in SP-MS (Placebo-controlled multic, 1998), it has not been confirmed by the following several clinical trials (Randomized controlled tri, 2001; Panitch et al., 2004; Andersen et al., 2004), leading to the a decline for the use of IFNb in SPMS. Mitoxantrone, an anthracenedione antibiotic with antineoplastic activity, showed a beneficial effect in a clinical trial with SPMS patients (Hartung et al., 2002), but the use of it in progressive MS has been gradually reduced in the clinic due to its poor safety profile. Simvastatin, a HMG-CoA reductase inhibitor, was effective in reducing the rate of brain volume loss and delaying the progression of disability in SPMS possibly due to its neuroprotective effects (Chataway et al., 2014). The clinical trial of biotin for progressive MS showed significant benefit in the primary endpoint that is due to enhancement of myelin repair and protection against axonal degeneration (Tourbah et al., 2016). As most of the successful drugs in SPMS described so far showed a clear general anti-inflammatory and/or neuroprotective effect targeting on the effector phase of the disease as a symptomatic treatment, fundamental therapies impacting on the pathophysiology of progressive MS are still elusive and exploratory research aiming for the development of radical treatment based on its pathogenic mechanisms is missing. In this regards, success of following two drugs shown to be effective for SPMS might provide a novel perspective not only for the development of new drugs, but also for unraveling the hidden pathogenic mechanisms of SPMS.

In 2016, ocrelizumab, an anti-CD20 monoclonal antibody, was demonstrated to have the ability to delay the accumulation of disability in patients with PPMS (Montalban et al., 2017). As several reports had already demonstrated a potential role of $CD20^+$ B cells in the pathogenesis of progressive MS (Serafini et al., 2004; Magliozzi et al., 2010; Howell et al., 2011), the result re-acknowledged the pathogenic involvement of B cells that accumulate in the subarachnoid space and meningeal follicles of patients with progressive MS (Li et al., 2018). Furthermore, as mentioned above, a beneficial effect of siponimod (BA312) has been demonstrated in SPMS patients (Gajofatto, 2017). Intriguingly, fingolimod, a drug with a similar profile to that of siponimod, failed to show efficacy in PPMS (Lublin et al., 2016). All these recent successes imply an arrival of a new era for drug development for progressive MS, as the efficacy of drugs in individual clinical trials will provide us with valuable information, not only for the development of novel therapeutics with similar pharmacological properties, but also for unveiling pathogenic mechanism lurking behind the superficial neurodegeneration emerged in progressive MS. Although the involvement of acquired immunity is usually underestimated as a cause of progressive MS, we might need to reconsider the sphingosine 1-phosphate receptor-mediated dynamic action of immune components and the pathogenic functions of residual T cells and B cells infiltrated in the diseased CNS.

4. Novel mechanism associated with acquired immunity and relevant biomarker for chronic progressive MS

4.1. Acute experimental autoimmune encephalomyelitis (EAE) and RR-MS are controlled by NR4A2-dependent Th cells

Animal models for human diseases such as MS provide a great opportunity to gain clear-cut insights into underlying pathogenic mechanisms of the disease. The most well accepted animal model for MS is EAE (Croxford et al., 2011; Constantinescu et al., 2011), where mice are immunized with myelin peptides derived from MOG, PLP etc. emulsified in complete Freund's adjuvant (CFA) to induce differentiation of autoreactive effector T cells. Once these encephalitogenic T cells infiltrate into the CNS, they secrete inflammatory cytokines, including IL-17 and IFN-γ, and recruit other inflammatory immune cells to promote damage to myelin and induce ascending paralysis, a typical signs of EAE.

Previously, we identified NR4A2 as one of the orphan nuclear receptors that is selectively upregulated in $CD4^+$ T cells derived from RRMS patients (Doi et al., 2008; Satoh et al., 2005). Interestingly, upregulation of NR4A2 is reproducible in $CD4^+$ T cells infiltrating the CNS and in peripheral blood of mice with EAE (Doi et al., 2008; Raveney et al., 2013). Further analysis revealed that NR4A2 plays a critical role in the expression of genes associated with the development of Th17 cells such as IL-17, IL-23 receptor and IL-21 by Th cells without affecting their expression of RORγt (Raveney et al., 2013). In addition, Systemic treatment with NR4A2-specific siRNA reduced IL-17 production by effector Th cells and protected mice from EAE signs.

Conditional knockout (cKO) mice of NR4A2 gene (NR4A2cKO mice) develop significantly ameliorated disease together with reduced accumulation of Th17 cell in the CNS, suggesting the crucial role of NR4A2 on the development of EAE (Raveney et al., 2015). Unexpectedly, NR4A2cKO mice developed late EAE-like signs, suggesting that clinical stages of MOG35–55-induced EAE in C57BL/6 mice can be separated into two phases: an NR4A2-dependent early/acute phase and an NR4A2-independent late/chronic phase (Fig. 1).

4.2. Late EAE and SP-MS are controlled by Eomes-expressing novel Th cells

As passive transfer of Eomes + Th cells isolated from mice with late/chronic EAE induced unexpected rapid worsening of EAE after transfer into NR4A2cKO mice before the onset of late/chronic EAE, suggesting the existence of pathogenic component within Th cells

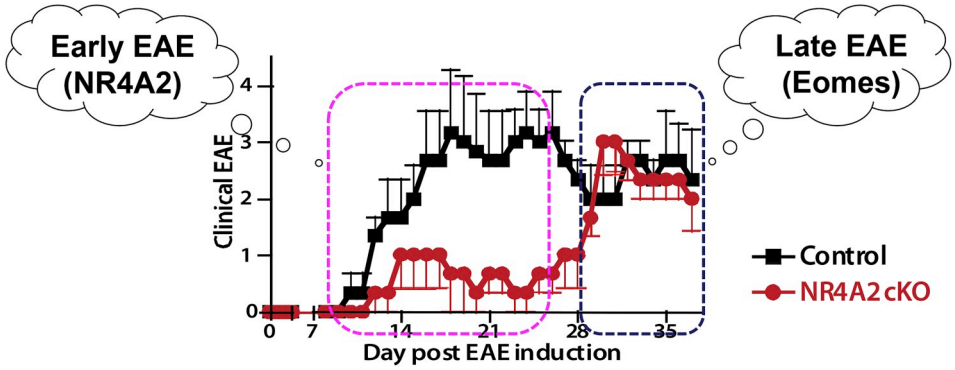

Fig. 1. EAE is composed of early (acute) EAE and late (chronic) EAE, which are mutually independent. Late (chronic) EAE is caused by Eomes-positive Th cells and suppression of Eomes has therapeutic effect against late EAE. Importantly, late (chronic) EAE is concealed by prolonged signs of early EAE in mice sufficient for NR4A2 gene.

obtained from mice with late/chronic EAE. In brief, transfer of $CD4^+$ T cells isolated from the CNS of control or NR4A2 cKO mice at late/chronic EAE phase (less than 50,000 activated T cells per head) into NR4A2 cKO mice before the onset of late disease (clinical score 1.0) over the following week induced a very rapid worsening of EAE in the recipient mice (Raveney et al., 2015). In the late/chronic phase of EAE, we identified that CNS-infiltrating Th cells showed higher expression of the Eomes gene (Raveney et al., 2015) and, those helper T cells from late/chronic EAE require Eomes expression to exert pathogenic activity in vivo, as similar transfer of CNS helper T cells obtained from NR4A2/Eomes DKO mice failed to induce clinical symptoms of EAE in recipient mice (Raveney et al., 2015). Accordingly, Eomes cKO mice immunized with MOG35–55 showed reduced EAE symptoms during the late/chronic stage, although the pathogenic roles of Eomes during early/acute EAE are not well analysed yet and are still controvertial (Raveney et al., 2015; Stienne et al., 2016). Although expression of Eomes gene and accumulation of Eomes-expressing helper T cells are relatively higher in NR4A2 cKO mice, possibly due to compensatory effect of helper T cells that failed to differentiate into pathogenic IL-17-producing cells due to the lack of the NR4A2 gene, these results suggest that the pathogenic roles of NR4A2 and Eomes are fundamentally irrelevant, suggesting that pathogenesis of early/acute EAE and late/chronic EAE are mutually independent. Therefore, we have successfully demonstrated that superficially-monophasic EAE induced by immunization of MOG35-55 peptide into C57BL/6 mice is composed of at least two independent early/acute and late/chronic disease and this cryptic late/chronic disease is noticeable only when early/acute disease is blocked in NR4A2 cKO mice.

The immunological functions of Eomes were originally described through selective expression of the molecule in $CD8^+$ cytotoxic T cells (CTLs) (Pearce et al., 2003) and maturation and effector function of natural killer cells are also regulated by Eomes (Gordon et al., 2012). Overexpression of Eomes in T cell exert preferential upregulation of granzyme B and perforin, two key component involved in cytolytic activity of CTLs. Intriguingly, reciprocal regulation of Eomes expression and Th17 cell differentiation were described (Ichiyama et al., 2011), again suggesting that pathogenesis of early/acute EAE and late/chronic EAE are mutually independent. The multifaceted role of Eomes has been described in the homeostasis of central memory T cells (Banerjee et al., 2010), regulation of innate-like T cells in the thymus (Gordon et al., 2011) or virtual memory T cells in the periphery (Park et al., 2016), and tissue-resident memory T cells (Mackay et al., 2015). In relevance to the pathogenic roles of T cells, cytotoxic helper T cells with high expression of Eomes have been described within tumor-infiltrating T cells after systemic administration of anti-4-1BB antibody (Curran et al., 2013) or after persistent viral infection (Marshall and Swain, 2011; Takeuchi and Saito, 2017). Of note, tumor microenvironments and chronically infected tissues provide strongly associated microenvironments of chronic inflammation that are commonly observed in SP-MS as well.

Although Eomes expression by Th cells in the context of autoimmune inflammation has not been previously described, accumulation of Eomes + Th cells in the CNS was persistent and stable over 15 weeks after induction of EAE. Then, we applied Eomes-specific siRNA in vivo and revealed that systemic administration of Eomes-specific siRNA significantly ameliorated the severity of late/chronic EAE. Furthermore, we successfully demonstrated that late/chronic stage of EAE was markedly reduced in NR4A2/Eomes DKO mice. Therefore, the development of late/chronic EAE was promoted by pathogenic Eomes + Th cells infiltrating the CNS.

Further analysis on frequency of Eomes + Th cells in peripheral blood mononuclear cells (PBMCs) revealed that healthy controls (HC), or patients with either RRMS contains 2%–12% of Eomes + Th cells within total $CD4^+$ T cells, which was consistent with a recent report (Knox et al., 2014). In contrast, the proportion of Eomes + Th cells was significantly increased in patients with SP-MS, showing the striking resemblance with late/chronic EAE. Importantly, accumulation of Eomes + Th cells was not correlated with patients' background such as age, gender, treatment, or current status of disability in individual patients. Proportions of Eomes + Th cells were further enriched in the CSF from patients with SP-MS, indicating their propensity for moving to the site of autoimmune inflammation. Therefore, Eomes + Th cells might also play a key pathogenic role in SP-MS.

Mouse and human Eomes + Th cells from SP-MS patients are revealed to express cytotoxic markers granzyme B and exhibit mobilization of CD107a, also known as lysosomal-associated membrane protein 1 (LAMP-1) upon stimulation, sharing phenotype with conventional cytotoxic T cells. Further, as demonstrated in recent report, perforin-independent mechanism of cytotoxicity (Wang et al., 2012) seems to be involved in neuronal cell death. Accordingly, administeration of granzyme B-specific siRNA or specific inhibitors for protease-activated receptor (Par)-1 significantly inhibited the development of late-chronic EAE, implying that the perforin-independent mechanism of neuronal death can be mediated by granzyme B/Par-1 interactions ignited by infiltrating Eomes + Th cells. These data raise the possibility that granzyme B-releasing Eomes + Th cells may help to solve the cellular and molecular mechanisms of neurodegeneration associated with pathogenesis of SP-MS (Fig. 2).

5. Future perspective

The long history of MS study with a numerous publications certifies the usefulness of EAE for investigating pathogenic mechanism of MS (Gold et al., 2006; Man et al., 2007; Pierson et al., 2012). However, it is surprising that MOG35-55 peptide-induced monophasic signs of EAE in C57BL/6 mice, the most commonly used model of EAE is composed of at least two independent pathomechanisms. As mentioned above, early EAE is NR4A2-dependent, Th17 cell-mediated acute disease, resembling the pathogenesis of RR-MS. Intriguingly in the absence of NR4A2 in Th cells, mice develop another form of late disease that is Eomes-dependent, which shows significant overlap in the pathogenesis of chronic CNS inflammation associated with persistent and progressive

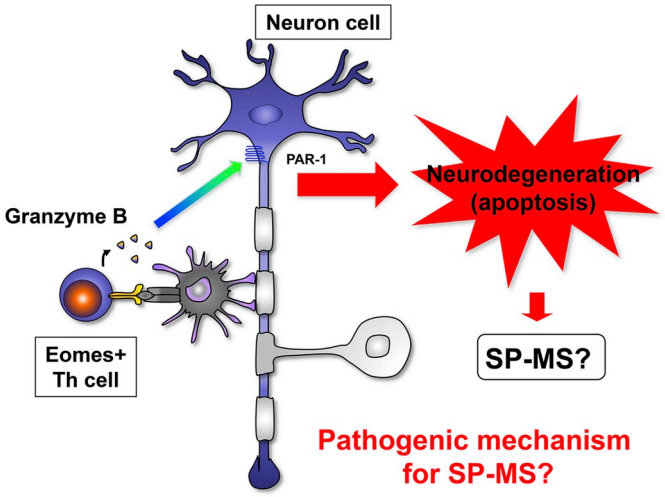

Fig. 2. Hypothetical pathogenic mechanisms for SPMS.
Eomes + Th cell-derived granzyme B may activate Par-1 expressed on neurons, possibly leading to neurodegeneration associated with pathogenesis of SP-MS.

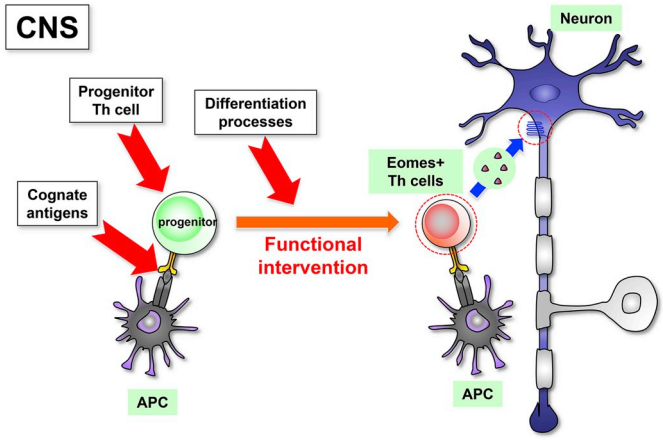

Fig. 3. Potential therapeutic targets for SP-MS upstream of Eomes + Th cell development.
The differentiation mechanism, cognate antigen, and Eomes + Th cell progenitors in the CNS will provide additional therapeutic intervention targeting Eomes + Th cells for preventing transition of the disease from RR-MS to SP-MS.

neurological dysfunctions typically observed in SP-MS (Raveney et al., 2015). As summarized in this review article, we have successfully established another scenario for the pathogenesis of SP-MS, in which Eomes + Th cells, a previously unappreciated helper T cell subset with cytotoxic potential for neurons, play an essential role for development of the disease (Raveney et al., 2015). Interestingly, large genome-wide association studies (GWAS) have demonstrated a significant link between Eomes gene polymorphism and the development of MS (Sawcer et al., 2011). As the pathogenic or protective involvement of T cells has been reported in diverse neurodegenerative diseases (Anderson et al., 2014), we believe that this novel finding could be a breakthrough for understanding neurodegenerative symptoms observed not only in SP-MS, but also in other neurodegenerative diseases. Importantly, this late disease is detectable only in the absence of NR4A2 in mice, because prolonged early disease conceals the concomitantly established late disease in B6 mice, suggesting that EAE in NR4A2cKO mice provides a novel tool for analyzing pathogenesis of late EAE and SP-MS.

Although a limited efficacy of immunomodulatory drugs could be interpreted by an active involvement of innate immunity rather than acquired immunity (Weiner, 2009), recent progress in the clinical trials of Ocrelizumab for PP-MS (Gajofatto, 2017) and Siponimod for SP-MS (Gajofatto et al., 2017) strongly implicate the involvement of immune components of acquired immunity such as T cells and B cells. Furthermore, pathological studies emphasized the presence of ectopic lymphoid follicles that is strongly associated with SP-MS (Magliozzi et al., 2007, 2010; Howell et al., 2011). A clear gradient of neuronal loss is observed in grey matter lesions and normal-appearing grey matter in the motor cortex of follicle-positive SP-MS cases, suggesting that cytotoxic molecules diffusing from the meninges participate in grey matter pathology and the following worsening of clinical disability (Magliozzi et al., 2010). A novel pathogenic subset of helper T cells (Eomes + T cells) is likely to be a strong candidate as a source of such cytolytic factors. Thus, targeting Eomes + Th cells and the effector molecules such as granzyme B and PAR-1 may yield specific treatments for SP-MS.

The in vivo origin of Eomes + Th cells is still controversial. Recent studies have revealed that Th cells with similar Eomes-expressing phenotype are generated under chronic inflammatory conditions associated with chronic viral infection such as CMV and HIV-1 or can be induced within tumor microenvironments by immunomodulation (Curran et al., 2013; Marshall and Swain, 2011; Takeuchi and Saito, 2017). Therefore, the developmental processes of Eomes + Th cells in the CNS under chronic inflammation will provide other therapeutic means for preventing transition of the disease from RR-MS to SP-MS (Fig. 3). Importantly, Eomes-expressing T-helper cells described in this manuscript are observed only in the context of in vivo pathology of SPMS and we have never successfully obtained these cells in in vitro differentiation conditions. Therefore, they will never be considered similar to established subsets of T cells such as Th1 or Th17 cells and are to be argued only within their relevance to the pathogenesis of SP-MS. Although inflammatory conditions might able to induce Eomes expression in T cells under specific circumstances, they could be different in the brain under SP-MS conditions, tumor microenvironments and during chronic viral infection.

Ectopic lymphoid follicles formed in the leptomeninges during the course of transition and disease progression may provide an effective scaffold allowing stable interaction between T cells, B cells, a variety of myeloid cells and dendritic cells, leading to the generation of Eomes + Th cells in the CNS of SP-MS patients. Recently, we investigated an intrinsic involvement of MHC class II-positive antigen-presenting cells (APCs) for the development of Eomes + Th cells in inflamed CNS and revealed that prolactin ectopically produced by CNS-infiltrating APCs plays a critical role for induction of Eomes expression in Th cells. Therefore, prevention of Eomes + Th cell generation could be considered for preventing transition of the disease from RR-MS to SP-MS. Classification criteria for progressive MS were recently revised (Lublin et al., 2014) and divided into four categories with two-dimensional parameters of active inflammation and clinical disease progression. Although active inflammation reflects the presence of clinical relapses or MRI-based symptoms, imaging techniques are not necessarily sensitive enough to detect smoldering CNS inflammation in SP-MS patients and EDSS score is not incisive enough to estimate the degree of ongoing disease progression. Therefore, it is a pressing issue to identify and determine novel biomarkers that reflect ongoing disease progression in MS. I hope that the frequency of Eomes + Th cells and the related parameters that could provide a novel measure effective for diagnosis of progressive MS.

Currently, further investigation is underway to fully understanding the pathogenic mechanisms of SP-MS and comprehensive molecular machinery of chronic neuroinflammation. Eomes + Th cells and the related cellular component accumulated in the CNS could provide promising candidates for development of novel therapeutic interventions for SP-MS.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.11.023.

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