Wang, Weikan, Aging Impairs Regulatory T cells to Affect the Mouse Model of Late-onset Multiple Sclerosis. Doctor of Philosophy (Biomedical Sciences), June 2022, pp. 110, 3 tables, 27 illustrations.

Summary

Although multiple sclerosis (MS) primarily onsets in young adults, it can also develop in the elderly, which is termed late-onset (aged) MS. $CD4^+Foxp3^+$ regulatory T (Treg) cells play an ameliorative role in severity of MS or its animal model experimental autoimmune encephalomyelitis (EAE), and the aged immune system accumulates peripheral Treg (pTreg) cells. However, late-onset MS in the aged patients presents a more progressive disease course. We investigated why the accumulated pTreg cells fail to ameliorate the MS severity in the aged individuals by using an aged EAE mouse model to recapitulate late-onset MS in patients. We observed that the onset of EAE is delayed in aged mice, but disease severity is increased compared to young EAE mice. We found that the distribution of Treg cells in aged EAE mice exhibited an increased proportion of polyclonal (pan-) pTreg cells and a decreased proportion of antigen specific-pTreg cells in the periphery, but decreased proportions of both pan- and antigen specific-Treg cells in the central nervous system (CNS). Transiently inhibiting Foxp3 or depleting pTreg cells partially corrected Treg distribution and restored the balance of effector T cells (Teff) and Treg cells in the aged inflamed CNS, thereby ameliorating the disease in the aged EAE mice. Furthermore, in the aged inflamed CNS, CNS-Treg cells exhibited a high plasticity and T effector (CNS-Teff) cells presented a great clonal expansion, disrupting the Treg/Teff balance. These results provide evidence and mechanism that accumulated aged pTreg cells play a detrimental role in neuronal inflammation of aged MS.

AGING IMPAIRS TREG CHARACTERISTICS TO AFFECT THE MOUSE MODEL OF LATE-ONSET MULTIPLE SCLEROSIS

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AGING IMPAIRS REGULATORY T CELLS TO AFFECT THE MOUSE MODEL OF LATE-ONSET MULTIPLE SCLEROSIS

DISSERTATION

Presented to the Graduate Council of the

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In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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LIST OF ABBREVIATIONS

AD: Alzheimer's disease

Aire: autoimmune regulator

APCs: antigen presenting cells

BBB: blood–brain barrier

CD: cluster of differentiation

CDRs: complementarity-determining regions

CNS: central nervous system

CP: choroid plexus

CSF: cerebrospinal fluid

cTECs: cortical thymic epithelial cells

DP: double positive

DPI: days post immunization

DT: diphtheria toxin

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein-Barr virus

ETPs: early T cell progenitor

Fezf2: FEZ family zinc finger protein 2

GO: Gene Ontology

HSC: hematopoietic stem cells

IFN: interferon

IL: interleukin

i.p. : intraperitoneally

LFB: Luxol Fast Blue

LN: lymph node

MBP: Myelin basic protein

MFI: Median florescence intensity

MHC: histocompatibility complex molecule

MOG: myelin oligodendrocyte glycoprotein

MRI: magnetic resonance imaging

MS: multiple sclerosis

mTECs: medullary thymic epithelial cells

NKR: natural killer cells receptor

OPCs: oligodendrocyte precursor cells

pan-: polyclonal

PBMC: peripheral blood mononuclear cells

PLP: proteolipid protein

PPMS: primary progressive multiple sclerosis

PRMS: progressive relapsing multiple sclerosis

pTreg: peripheral regulatory T cell

RQ: relative quantitative

RRMS: relapsing remitting multiple sclerosis

SAS: subarachnoid space

SASP: senescence-associated secretory phenotype

s.c.: subcutaneous

sc-RNA-Seq: single-cell RNA sequencing

SP: single positive

SPMS: secondary progressive MS

Tcon: conventional T cell

TCR: T-cell receptor

TECs: thymic epithelial cells

Teff: effector T cell

TF: transcription factor

Th: helper T cell

TM: memory T cell

TNF: tumor necrosis factor

Treg: regulatory T cell

t-SNE: t-distributed stochastic neighbor embedding

tTreg: thymus-derived regulatory T cell

WT: wild-type

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ATTRIBUTION OF PUBLISHED WORK

Permission was obtained to utilize my previously published work as part of this Dissertation as long as it was clearly indicated. Therefore, here are the sections taken directly from my published manuscripts:

CHAPTER I. Section 1.2.2 including Figure 1 is adapted from my published review manuscript:

Thomas R, Wang W, Su DM. Contributions of Age-Related Thymic Involution to Immunosenescence and Inflammaging. Immun Ageing. 2020 Jan 20;17:2. doi: 10.1186/s12979020-0173-8. PMID: 31988649; PMCID: PMC6971920.

CHAPTER II. Figure 5, Figure 6 and Table 5 are adapted from my published research manuscript:

Wang W, Thomas R, Oh J, Su DM. Accumulation of pTreg cells is detrimental in late-onset (aged) mouse model of multiple sclerosis. Aging Cell. 2022 Jun;21(6):e13630. doi: 10.1111/acel.13630. Epub 2022 May 26. PMID: 35615905

CHAPTERS III, IV, V. All sections and figures are adapted from my published research manuscript:

Wang W, Thomas R, Oh J, Su DM. Accumulation of pTreg cells is detrimental in late-onset (aged) mouse model of multiple sclerosis. Aging Cell. 2022 Jun;21(6):e13630. doi: 10.1111/acel.13630. Epub 2022 May 26. PMID: 35615905

Chapter I. Introduction

1.1 The immune system: innate and adaptive immunity

The immune system is comprised of immune cells and chemicals that protect an individual from infection, toxins, tumor cells (1, 2). The immune system can be divided into two lines of defense: innate immunity and adaptive immunity. Innate immunity is the first line of defense to an intruding pathogen used by the host to immediately response and clear a wide range of pathogens. Adaptive immunity, on the other hand, has a delayed immune response to pathogens. Most notedly, adaptive immunity has higher specificity to antigens than innate immunity and has the capacity of memory to more effectively clean up antigens in a subsequent exposure to the antigens (3). Innate and adaptive immunity are complementarily and synergically fight against abnormal antigens in the host and maintain the homeostasis (1, 2).

Innate immunity is comprised of four types of defensive barriers, including anatomic (skin and mucous membrane), physiologic (temperature, low pH and chemical mediators), endocytic and phagocytic, and inflammatory (4). Innate immune cells can rapidly produce cytokines and chemokines to recruit more immune cells to sites of infection and inflammation, thereby localizing and cleaning up pathogens. Numerous cells are involved in the innate immune response such as macrophages, dendritic cells, neutrophils, basophils, eosinophils, mast cells, natural killer cells and innate lymphoid cells (4). Among the innate immune cells, dendritic cells are the most specialized antigen presenting cells, which present processed antigens to T cells and therefore connect the innate immunity and adaptive immunity (5).

The initiation of adaptive immune response, which is aided by innate immune system, is critical when innate immunity is not sufficient enough to eliminate infectious agents or other intruding foreign antigens (4). T cells and B cells are the major cell populations of the adaptive immunity. The primary advantages of the adaptive immune response include a) a more specific recognition of non-self antigens using their antigen specific T cell receptors or B cell receptors; b) the development of an immunologic memory that can quickly eliminate a specific pathogen should subsequent infections occur (2); and c) maintaining immune tolerance to self-reactive damage (6, 7). In the following sections, T cells will be more specifically discussed and related with the development of a neuroautoimmune disease multiple sclerosis (MS).

1.2 T cells: development, differentiation and their potentials for CNS autoimmunity

The development of T cells, particularly referring to α/β T cells, is primarily in the thymus, which is the central and primary lymphoid organ for cellular immunity. T cell development and differentiation include intricate processes, such as T-cell receptor (TCR) rearrangement and positive and negative selection. The generation of TCR diversity and specificity by TCR gene rearrangement and the following positive and negative selection are two critical steps for the capacity of T cells to efficiently recognize pathogenic antigens along with immune tolerance for self-tissues (8-10). Any perturbation to the thymic selection will result in disorders in health. For example, if the central nervous system (CNS) antigen-specific effector T (Teff) cells escape from negative selection and are released to the periphery from the thymus, they could induce neuroinflammatory diseases by recognizing either the CNS abnormal antigens (e.g. amyloid β in Alzheimer's disease) (11) or CNS self-antigens (e.g. myelin sheath antigens in MS/EAE) (12, 13).

1.2.1 TCR Rearrangement for TCR Specificity and Diversity

The early stage of thymocyte (immature T cell) development is featured with gene arrangement for TCR (we focus on the α/β TCR in this dissertation) diversity and specificity. TCR specificity of each T cell and TCR diversity of the whole T cell repertoire are the fundamentals for the recognition of a broad spectrum of specific antigens in T immune responses. To generate productive TCRs, thymocytes undergo somatic V(D)J recombination of TCR genes to encode TCRα and TCRβ chains. In this process, the recombination activating genes (RAG), including RAG1 and RAG2, are the key enzymes that recruit V, D, and J coding sequences and facilitate recombination (9, 10). After rearrangement, the hypervariable regions or complementarity-determining regions (CDRs) of a TCR serves as the specific antigen-recognizing component (9, 10). The theoretical TCRαβ repertoire diversity can be around 10^{15} and 10^{20} in healthy adult mice and humans, respectively (14-16). However, one study found the functional splenic naïve αβTCR repertoire diversity of 1-2 x 10^6 in healthy adult mice (17), which can partially due to the positive and negative selection we will discuss later.

1.2.2 T cell Selection and Imperfect Central Tolerance for CNS Autoimmunity

After the generation of TCR for each thymocyte, further interactions between thymic epithelial cells (TECs) and immature T cells (or thymocytes) determine the mature fates of thymocytes. This involves signaling entering the immature thymocytes. One of the most important signaling is the positive selection signaling. This happens at the stage of double positive (DP, i.e. expression of CD4 and CD8 co-receptors) thymocytes. Survival of the DP thymocytes rely on their TCRs' recognition with histocompatibility complex class II molecule (MHC-II) or MHC-I

molecule expressed by cortical TECs (cTECs). This process determines development toward CD4, which is selected by MHC-II, or CD8, which is selected by MHC-I, single positive (SP) lineage (18-20).

Following the positive selection, a sufficient TCR diversity can be not only essential for a robust immune response to various foreign antigens, but also for self-tissue antigens. Therefore, it is important for $CD4^{SP}$ and $CD8^{SP}$ thymocytes to migrate to the thymic medulla for negative selection to eliminate these self-reactive thymocytes and for establishing central tolerance (19, 21). To take $CD4^{SP}$ thymocytes as an example, negative selection depends on avidity of the interaction between their TCRs and self-antigen (peptide)/ MHC-II complexes expressed by medullary TECs (mTECs). More specifically, 1) high avidity of the interaction between self-reactive TCRs and self-peptide/MHC-II complexes induces strong TCR signaling in SP thymocytes cells, leading to clonal depletion by negative selection; 2) low avidity of this interaction induces thymocyte differentiation basically into foreign antigen-specific conventional T (Tcon) cells, and 3) intermediate avidity of this interaction in the presence of IL-2 induces thymocyte differentiation into $CD4^{+}Foxp3^{+}$ thymus-derived Treg (tTreg) cells, which process is also termed agonist selection (Fig. 1) (22-26). Self-reactive Tcon cell depletion by negative section and $CD4^{+}Foxp3^{+}$ tTreg generation via agonist selection are the two mechanisms of the generation of T cell central tolerance in the thymus to minimize and control autoimmunity (27). Of note, autoimmune regulator (*Aire*) gene and FEZ family zinc finger protein 2 (*Fezf2*) gene are two critical transcription factors (TFs) regulating promiscuous expression of self-antigen by mTECs for central tolerance (28-30). Notably, *Aire* gene defect causes severe autoimmunity, such as autoimmune polyendocrineopathy-candidiasis-ectodermal dystrophy (31).

Finally, these subsets of $CD4^{SP}$ naïve T cells including most numbers of foreign antigen-specific Tcon cells, small numbers of self-reactive Tcon cells, and self-recognizing tTreg cells migrate to peripheral lymphoid tissues. In the peripheral lymphoid tissues (secondary lymphoid tissues), certain foreign antigen-specific Tcon cells and the self-reactive Tcon cells could potentially recognize the CNS abnormal antigens (e.g. amyloid β in Alzheimer's disease) (11) or CNS self-antigens (e.g. myelin sheath antigens in MS/EAE) (12, 13). The escape of myelin-specific Tcon cells from deletion in the thymus can be attributed to low affinity of myelin-specific TCRs, and/or low-expressed myelin peptide/MHC-II complex by mTECs. This was confirmed by T cells specific for different epitopes of myelin basic protein (MBP) in B10.Pl mice, whose $MBP_{121–140}$ and MHC-II forms a stable complex and induces depletion, but $MBP_{Ac1–11}$ and MHC-II forms an unstable complex to fails their depletion (32). However, the CNS parenchyma expresses abundant myelin antigens that can be effectively presented by antigen presenting cells (APCs) to Tcon cells bearing the low-affinity myelin-specific TCRs, thereby, these myelin-specific Tcon cells can be activated. As the function of negative selection is decreased with thymic aging, more CNS-antigen specific T cells might escape from negative selection and they are released to the periphery for CNS autoimmunity (33). Notably, another mechanism of central tolerance is to generate tTreg cells, and tTreg cells migrate to the periphery and suppress the self-reactive Tcon cell-mediated autoimmunity (34). However, the efficiency of this mechanism can be declined with thymic involution with aging, because as found in our recent study, the involved thymus may create "holes" in tTreg clone repertoire for self-antigens (35). This may fail the Treg cells' recognition of certain self-antigens that are recognized by self-reactive Tcon cells, leading to an imbalanced activation of Tcon versus Treg cells and losing control of autoimmune response.

Therefore, the skewed tTreg TCR repertoire can be a potential contributor to increased CNS autoimmunity in the aged individuals, which is a focus of this project.

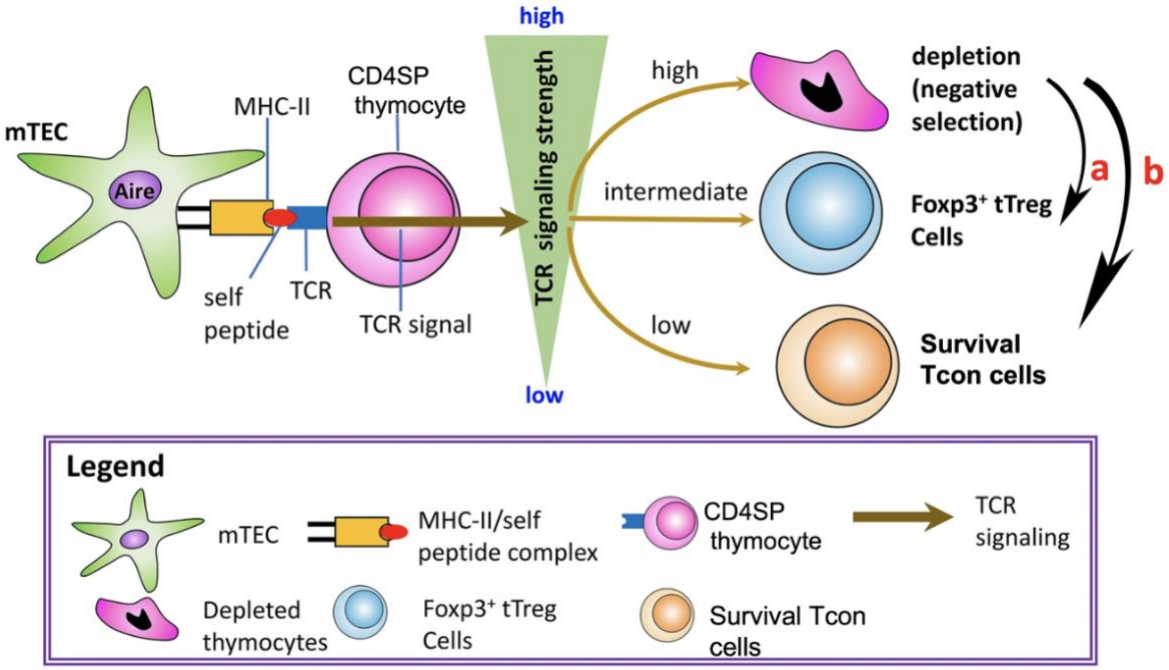


Figure 1. TCR signaling strength decides CD4^{SP} thymocyte clone fates. Interaction between self-peptide/MHC-II complex on mTEC and self-reactive TCR on CD4^{SP} thymocyte produces three types of signaling strength: (1) a strong signal leads to negative selection, resulting in thymocyte depletion; (2) an intermediate signal leads to tTreg generation; (3) a weak signal results in thymocyte differentiation into Tcon cells. Aged involuted thymus has reduced self-antigen presentation by mTECs, which shifts signaling strength from strong to intermediate and relatively enhances polyclonal tTreg generation (black arrow—a); while in some cases, antigen-specific interactions exhibit an even weaker signal, resulting in diminished antigen-specific tTreg cells and increased antigen-specific Tcon cells (black arrow—b). (Adapted from *Rachel Thomas, Weikan Wang, Dong-Ming Su. Contributions of Age-Related Thymic Involution to Immunosenescence and Inflammaging. Immun Ageing. 2020 Jan 20;17:2.*)

1.3 Multiple Sclerosis

Multiple sclerosis (MS) is a T cell-mediated autoimmune neuroinflammatory disease, and it is affecting millions of people worldwide (36). MS is characterized by the demyelination of axons of neurons in the central nervous system (CNS) and it progresses into the loss of axons, leading to neurological dysfunctions (37). The etiology and mechanism of MS onset and severity are not yet fully understood. One of the accepted mechanisms is that the initial contact of certain viral antigens or superantigens with molecular similarity (mimicry) with some CNS myelin antigens elicit $CD4^+$ helper T (Th) cells and macrophages to produce proinflammatory cytokines including interferon (IFN)-γ, interleukin (IL)-17 and IL-1. These cytokines lead to productions of metalloproteinases, which breakdown the blood–brain barrier (BBB), and the activated Th cells up-regulate integrins such as VLA-4. Consequently, Th cells and other immune cells infiltrate into the CNS and induce neuroinflammation and demyelination (38-40).

The etiology of MS involves environmental exposure and genetic susceptibility. Two mostly investigated environmental factors associated with MS include high latitude regions and viral infections. Global distribution of MS is generally increased with the distance from the equator (41) and the north Europeans are the most susceptible population (42). Migrants from high-risk to low-risk regions in childhood have reduced risk for the disease and vice versa (43). Viral exposure is another important risk factor as infection with Epstein-Barr virus (EBV) at an early age increases the susceptibility to MS (44). The risk of a relapse is also increased after viral exposure, especially to upper respiratory gastrointestinal viral infections (45, 46). One of the well elucidated mechanisms for the association between EBV and MS is cross-reactivity by T cells recognizing both myelin antigen MBP and an EBV peptide (47). Other relevant environmental factors include low sun exposure, vitamin D insufficiency, physical and emotional stressors, air pollutants,

cigarette smoking, and toxins (48-52). MS has a familial aggregation, indicating the effect of genetic susceptibility to the disease (53). Primarily, MHC alleles DR15 and DQ6 have been reported as significant genetic risks for MS (54-56), and DR15 haplotype is considered to be the major genetic factor for northern European patients (55). Recent study has clarified a convincing mechanism regrading DR15 haplotype associated MS susceptibility. Basically, DR15 derived self-peptides HLA-DR-α-SPs and HLA-DR-β-SPs have molecule mimicry with EBV antigens, thus autoreactive T memory cells preactivated by HLA-DR-α-SPs and HLA-DR-β-SPs are highly activated when exposed to EBV. As these autoreactive memory T (TM) cells also recognize myelin antigen, the individuals are highly susceptible to MS (56).

MS patients can present almost any neurological symptoms dependent on the regions of demyelination and inflammation in the CNS, including cerebrum, optic nerves, cerebellum and cerebellar pathway, brain stem and different segments of the spinal cord. Among all the symptoms, autonomic, visual, motor, and sensory dysfunctions are the most common (43). The demyelination foci can by determined by magnetic resonance imaging (MRI), which shows multiple hyperintense lesions in T1 and T2-weighted images (57, 58). Initial inflammation in most patients is usually transient and it is followed by durable remyelination, thereby, neurological dysfunction can recover. However, the pathological changes become dominated with extensive microglial activation and chronic neurodegeneration, leading to progressive disability (43). The disease can evolve for several decades and lead to death in 2/3 cases due to the increased complications of infections with advanced neurological disability, and the median survival time after onset can vary from 28 years to 45 years (59-61). Based on the characteristics of the symptom severity and course progression, subtypes of MS were first formally defined by the U.S. National Multiple Sclerosis Society Advisory Committee depending on the disease courses (Table 1) (62) including:

(1) Progressive relapsing MS (PRMS): progressive disease from onset, with clear acute relapses, with or without full recovery;

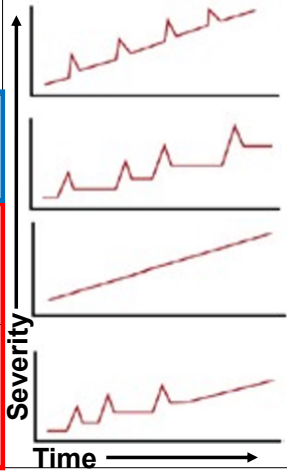
(2) Relapsing remitting MS (RRMS): episodes of acute worsening of neurologic functioning with total or partial recovery of disease;

(3) Primary progressive MS (PPMS): steadily worsening neurologic function from the beginning without any distinct relapses or remissions;

(4) Secondary progressive MS (SPMS): progressive course of neurologic dysfunction with or without relapses.

MS diagnosis includes medical history, MRI imaging, cerebrospinal fluid (CSF) analysis and evoked potentials. Obtaining a medical history should consider the onset of the first symptoms, disease course, food habits, and history of medications taken. MRIs can localize most demyelination lesions and tissue damages throughout the CNS. Evoked potentials test of visual, brain stem auditory, and somatosensory evoked potentials indicate demyelination in the optic nerve and specific areas of CNS. In addition, CSF analysis for MBP and IgG and blood sample analysis may be diagnostically helpful (63). Nevertheless, it is necessary to exclude other diseases that present similar symptoms or laboratory examination results to multiple sclerosis, including vascular diseases, spinal cord compression, vitamin B12 deficiency, and CNS infection (64).

Table 1. Clinic subtypes of MS and the onset frequencies in young and aged patients

Subtype	Patterns	Severity	% Onset in young patients	% Onset in Aged patients
Progressive-relapsing MS (PRMS)		+	**5%**	**~8%**
Relapsing-remitting MS (RRMS)		+	**80 – 85%**	**< 40%**
Primary progressive MS (PPMS)		+++	**10%**	**~29%**
Secondary progressive MS (SPMS)		++	**-**	**~26%**

1.4 T Cell-Mediated Mechanisms of MS pathology and Its Animal Model EAE

In order to have a better understanding of MS pathology, mouse EAE model is used for the research of T cell involved mechanism of MS (40, 65). EAE is induced by (1) activating myelin-specific T cells by immunizing naïve T cells with myelin proteins or peptides, including myelin oligodendrocyte glycoprotein (MOG), MBP, or proteolipid protein (PLP), which are expressed by the myelin sheath in the CNS, along with adjuvants capable of activating the innate immune system, and (2) increasing the permeability of the BBB by a systemic treatment of pertussis toxin (PT) (40, 66). This protocol mimics MS in terms of the myelin-specific T cells mediated neuroinflammation and demyelination. The major differences between MS and induced EAE are (1) EAE requires an external sensitization with a known CNS antigen (i.e. MOG, MBP, or PLP), whereas in MS no artificial sensitization is induced and the autoantigens are not unique; (2) the increased BBB permeability in MS is not induced by a determined toxin, but more likely induced by systemic inflammation. With this regard, EAE is a preferred model for immunological and pathological studies of MS, as in our current work, but it is not favorable for etiological study.

Before the onset of CNS pathology of MS/EAE, myelin-specific $CD4^+$ T cells, including Tcon and Treg cells, are firstly primed by antigens presented by APCs in the periphery. These antigens include myelin proteins/peptides that are injected into mice for EAE induction (66), environmental factors with molecule mimicry for myelin antigens (56), and myelin antigens from the CNS-draining cervical lymph nodes (67, 68). Activated myelin-specific $CD4^+$ Tcon cells are differentiated into Teff cells, including INF-γ producing Th1 cells and IL-17 producing Th17 cells, before migrating into the CNS (Fig.2) (69).

The barrier system of the CNS, specifically (1) the blood–brain barrier (BBB) surrounding parenchyma, and (2) the blood-cerebrospinal fluid (CSF) barrier surrounding the brain choroid plexus (CP) block the infiltration of most immune cells in the physiological state. However, activated T cells can cross the barriers due to their expression of adhesion molecules and chemokine receptors which interact with the barrier-expressed adhesion molecules and local chemokines in the inflammatory conditions (70). The subarachnoid space (SAS) is underneath the blood-CSF barrier, and it is the initial site where $CD4^+$ T cells infiltrate and get reactivated by MHC-II^+ APCs (71), and this initiation can be attributed to the constitutively highly expressed adhesion molecules at the CP (72). The expression of these adhesion molecules, especially ICAM-1 and VCAM-1, is regulated by local IFN-γ, which facilities the trafficking of peripheral immune cells into the CNS (73). The reactivation of $CD4^+$ T cells at SAS mediates inflammation augmentation and thereby activates vascular endothelial cells, which further recruits T cells into the perivascular space. Inflammation mediated by increased T cell activation in the SAS triggers the activation of proximal microglia, which causes distal microglia activation and upregulates adhesion molecules on parenchymal vasculature in broader areas for more infiltration of immune cells through the BBB and the brain-CSF barrier (74).

Initialization of MS/EAE is mediated by CNS-infiltrating $CD4^+$ Th1 and $CD4^+$ Th17 cells. Th1 cells requires IL-12 for differentiation and they are characterized by the secretion of IFN-γ (75, 76). The role of Th1 cells involved in MS pathology is determined by increased cytokine IL-12 and Th1 cells in the both the CSF and the inflamed CNS parenchyma of MS patients (77, 78). Furthermore, MS was exacerbated by the administration of IFN-γ (79). The role of Th1 cells in EAE is also supported by the IFN-γ producing Th1 cells in the CNS and the disease induction by adoptive transfer of Th1 cells (80, 81). However, it is striking that mice deficient in IL-12 and

IFN-γ also develop EAE (82-84), whereas IL-23 deficient mice are completely resistant to EAE (85). Given the crucial role of IL-23 for Th17 development (86) and the presence of Th17 infiltration into the CNS during EAE (87), CNS-infiltrated Th17 cells, which secret IL-17, are confirmed as another pathogenic T cell subsets for EAE induction. Further studies also suggested IL-17 and IFN-γ co-expressed $CD4^+$ T cells with MOG specificity contributed to EAE pathogenesis (88). IFN-γ and IL-17 contained inside the CNS serve as potent proinflammatory cytokines to activate CNS-resident innate immune cells including microglia and astrocytes (89-92). This activation can promote the disease in two ways: firstly, it stimulates the proinflammatory cytokine (IL-6, IFN-γ, IL-23, TNF-α, etc.) productions by microglia and astrocytes along with CNS-infiltrating macrophages to augment neuroinflammation (93-95), which induce demyelination followed by significant neuronal loss (96, 97); secondly, it upregulates MHC-II expression by microglia, astrocytes, and CNS-infiltrating macrophages, which facilitates myelin antigen presentation to Th cells for their further activation (98). In addition to the pathogenic process by Th1 and Th17 cells, $CD4^+Foxp3^+$ Treg cells which suppress proinflammatory T cells and other inflammatory immune cells (99) are regarded as protective cells (34, 100-105). Treg suppressive function involves multiple mechanisms via targeting to Teff cells or APCs (Fig. 3) (99). It was reported that transferring Treg cells into MOG-induced EAE mice attenuated disease (106). EAE severity was inversely related with the frequency of specific Treg cells in the inflamed CNS (107). In addition, another Th cell subset, Th2 cells, is also reported to be involved in EAE, but they play either protective (108) or pathogenic(109) roles, thus the role of Th2 cells in MS and EAE needs further investigations.

Although $CD4^+$ T cells are the traditional primary actors in MS/EAE pathogenesis and immunoregulation, $CD8^+$ T cells also infiltrate into the CNS of MS patients (110). Emerging

evidence shows that $CD8^+$ T cells are also involved in CNS autoimmunity (111, 112). These CNS-infiltrated $CD8^+$ T cells have either pathogenic (113, 114) or regulatory (112, 115) roles to either exacerbate or mitigate neuroinflammation during CNS autoimmunity (111, 112). Regarding MS/EAE immunopathogenesis, myelin-specific $CD8^+$ T cells exacerbate brain, though not spinal cord, inflammation via a Fas ligand-dependent mechanism to promote lesion formation in the brain (116). IFN-γ in the inflamed MS/EAE CNS upregulates MHC-I expression on oligodendrocyte precursor cells (OPCs), thereby amplifying OPCs' antigen presenting capacity to CD8 T cells in a positive feedback loop (117). In addition to inflammation-mediated myelin sheath damage, CD8 T cells may also directly induce demyelination, as MBP-specific CD8 T cell lines lyse oligodendrocytes in a co-culture system (118, 119). In addition, IL-17A secreting $CD8^+$ T cells, termed Tc17 cells, in the CNS support Th17 cell-mediated autoimmune encephalomyelitis (114). In the immuno-regulatory aspect, the role of $CD8^+$ T cells in MS/EAE remains controversial and the concept of the bona fide $CD8^+$ Treg subsets is not unanimously accepted (115, 120, 121). This is due to negligible numbers of $CD8^+Foxp3^+$ thymocytes (122), low proportion (<0.5% in mice) in a heterogenous peripheral T cell pool, uncertain phenotypic identity ($Foxp3^+$, $CD122^+$, $CD8\alpha\alpha^+$, etc.), and unclear regulatory mechanisms (via cytokines and/or direct killing?) (115, 123). Nevertheless, the suppressive function of $CD8^+$ Treg cells in MS/EAE disease has received attention (115, 120, 121, 124), and $CD8^+$ Treg cells potentially cooperates with $CD4^+$ Treg cells (125) in suppression of neuronal autoimmune inflammation (126).

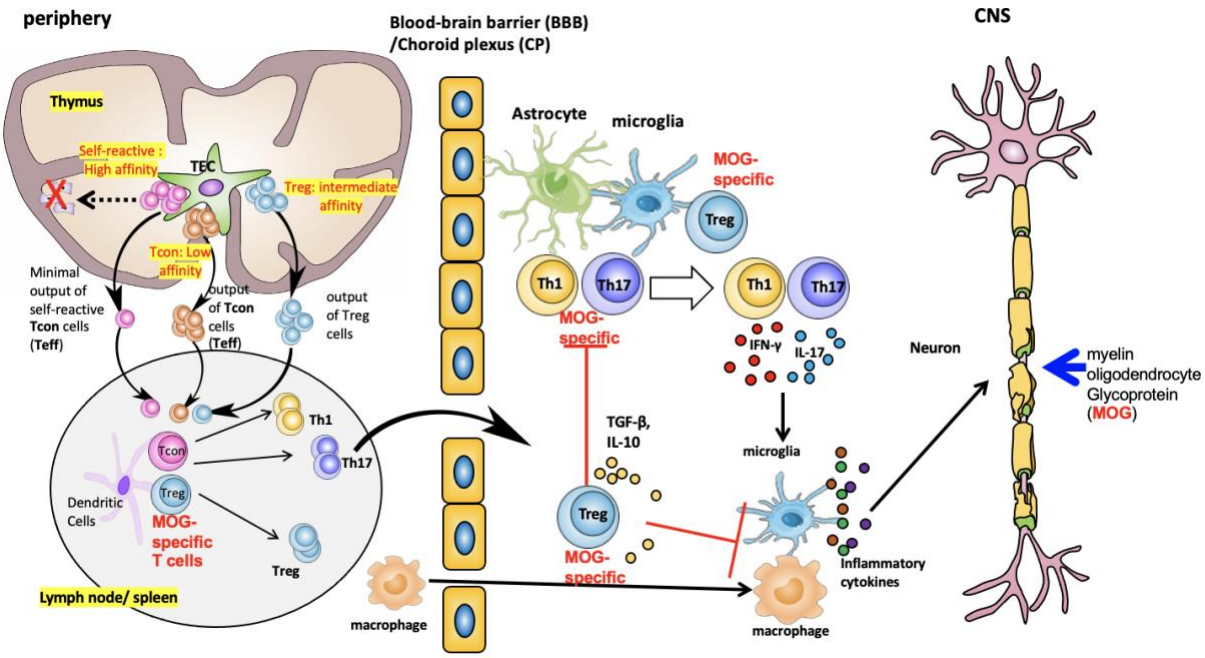


Figure 2. Participation of CD4$^+$ T cells in MOG induced EAE. Naïve CD4$^+$ T cells migrate from thymus to the periphery after selection. Upon the stimulation of myelin antigen (e.g. MOG) presented by APCs, MOG-specific Tcon cells and Treg cells are activated, among which MOG-specific Tcon cells are differentiated into effector T (Teff) cells, including INF-γ producing Th1 cells and IL-17 producing Th17 cells, before migrating into the CNS. Due to the increased permeability of BBB and CP, Th1, Th17 and Treg cells infiltrate into the CNS, along with other immune cells. Inside the CNS, Teff and Treg cells are reactivated by MOG antigen presented by CNS-resident APCs, including astrocytes and microglia. As a result, highly activated Teff cells secret large amounts of proinflammatory cytokines, especially INF-γ and IL-17 to mediated neuroinflammation, which leads to the demyelination of neuronal axons. However, Treg cells suppress the effector function of Teff cells and other proinflammatory immune cells to alleviate neuroinflammation.

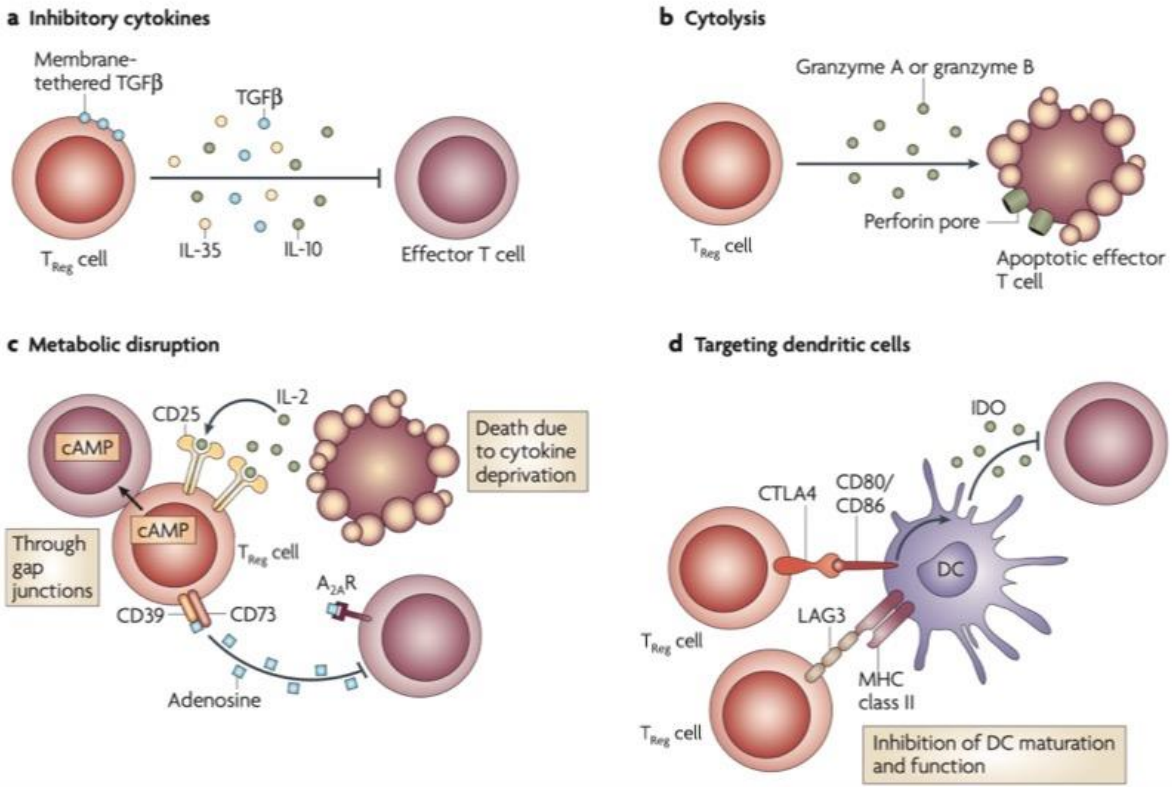


Figure 3. Basic mechanisms of Treg suppression. (a) Inhibitory cytokines IL-10, IL-35 and TGF-β to suppress Teff cells. **(b)** Granzymes and perforin-dependent cytolysis of Teff cells. **(c)** Metabolic disruption includes high-affinity CD25 (IL-2Rα)-dependent cytokine deprivation-mediated apoptosis, cAMP-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A ($A_{2A}R$)-mediated immunosuppression. **(d)** Targeting dendric cells (DCs) that modulate DC maturation / function such as lymphocyte-activation gene 3 (LAG3)–MHC-class-II mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs. (Adapted from *Dario A A Vignali , Lauren W Collison, Creg J Workman. How regulatory T cells work. Nat Rev Immunol . 2008 Jul;8(7):523-32.*)

1.5 Late-onset MS in aged patients

The ages of onset for most cases of MS range from 20 to 40 years old, therefore, most studies (on etiology, pathology, immunology, disease courses, etc.) have focused on young MS patients or used young mouse (2-3 months old) EAE models. However, late-onset MS whose manifestation occurs after the age of 60 years, has been reported in the elderly (127-130). In addition, the mean age of the MS population is rising (128). It was reported that 14% of MS patients aged 65 years and older in 2010 (131). Aging is a significant factor that shapes the MS disease course and correlates with increased disease comorbidities (128), such as cardiovascular comorbidities (132) and greater disability (133). In addition, aging also remodels MS disease types or courses (Table 1). Patients over the age of 65 are more likely to have severe progressive courses, with 29% patients having primary progressive MS (the disease progresses without remissions, degree of severity is defined as "+++"). 26% having second progressive MS (progression of the disease causes irreversible damage and disability, degree of severity is defined as "++"), and 8% with progressive relapsing MS (disease progresses with acute relapses, with or without full recovery, degree of severity is defined as "+"). However, most (about 80%) of the young patients have a relapsing-remitting MS (RRMS) course (degrees of severity is defined as "+") (Table 1) (128, 133). When compared to young mice, middle-aged mice have a delayed EAE onset, but after onset, the disease showed more severe outcomes than the young onset (134). This compelling evidence shows that pathological severity and disease course of MS/EAE disease in young and old individuals are distinctively different. Therefore, the underlying mechanism of the severity and disease course of late-onset (aged) MS remains to be investigated, especially T cell mediated mechanisms, for the development of therapeutic strategies.

1.6 Characteristics of the aged T cell immune system

Generally, immunity in the elderly exhibits decreased normal immune responses (i.e. immunosenescence) to pathogens, leading to increased vulnerability to infectious diseases and cancers, and decreased response to vaccines (135-137). One of the major players in immunosenescence is the senescence of T cell immune system, which is characterized by decreased immune responses to infections. The other major player is *increased* immune responses to self-tissues, which is a contributor to inflammaging (138). Inflammaging refers to increased abnormal non- or specific-immune responses to self-tissues (hyper-self-reactivity), leading to chronic sterile, low-grade inflammation. These two extremes: *immunosenescence and inflammaging synergistically* result in progressive dysfunction and autoimmunity (139).

Age-related T cell immune system remodeling includes the following aspects: (1) age-related thymic involution leading to decreased newly generated naïve T cells (140) and increased output of autoreactive T cells (33); (2) enhanced thymic Treg generation (141), along with TCR holes created in the Treg pool (35); (3) accumulated peripheral Treg cells (142-145); (4) Senescent T cells involved senescence-associated secretory phenotype (SASP) (146); (5) Increased clonal expansion of memory T cells with functional exhaustion (147).

These dysfunction via the T cell immune system remodeling primarily begins with the atrophied thymus (148). Age-related thymic involution is basically attributed to (1) deficit of hematopoietic stem cells (HSCs) leading to insufficient early T cell progenitor (ETP) entry into the aged thymus (149, 150), and (2) disruption of thymic stromal cell homeostasis, mainly TECs, especially due to the declined *FoxN1* expression by TECs with aging (151, 152). Thymic involution results in declined thymic output of newly generated naïve T cells and restricted TCR diversity (139, 140, 153), which causes a compromising the detection pathogens and poor

vaccination efficacy (135-137). With deep sequencing approach, TCR diversity in general has been confirmed to be decreased linearly with age in humans (154). More notedly, TCR diversity can be skewed in clonal size distributions, which may explain the susceptibility to certain age-related disorders (155). Our recent studies have found that the involuted thymus is more prone to generate autoreactive T cells (33) and fails to select certain antigen specific Treg clones (35). This skew in TCR clones is attributed to impaired negative selection of thymocytes due to dysfunctional mTECs in the aged involuted thymus. To take $CD4^{SP}$ thymocytes as an example, aged mTECs have impaired capacity for self-peptide/MHC-II ligand expression due to the decreased expressions of *Aire* gene and MHC-II for self-peptide presentation (33, 141, 148), therefore, a strong signaling strength mediated by high affinity TCR in self-reactive T cells shifts either to an intermediate strength, which favors $CD4^{SP}$ $Foxp3^{+}$ tTreg cell generation (Fig. 1, arrow-a), or to a low strength, which generates Tcon cells with self-reactivity (Fig. 1, arrow-b). As a result, self-reactive T cells may escape from depletion by negative selection and migrate into the periphery. Self-reactive T cells lead to tissue damage, which results in a sterile, low-grade, chronic inflammatory condition, termed "inflammaging" (33, 156, 157). Therefore, in the aged T cell immune system, the decreased normal immune response and enhanced self-reactivity comprise two sides of the same coin (138).

Treg cells are accumulated in the periphery of the age mice and humans. This is attributed to two mechanisms: (1) enhanced tTreg generation by aged thymus allows relatively increased Treg output to the aged periphery (141), and (2) progressive downregulation of pro-apoptotic *Bim* expression by Treg cells with age supports Treg survival in the periphery (142, 144). Although Treg cells are crucial for maintaining peripheral tolerance, since they play a vital role in control autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis (158) , type-I

diabetes (159), and MS/EAE (160, 161), ample evidence shows that accumulated pTreg cells have more disadvantages than advantages. This evidence came from at least in three types of diseases: a) In immune responses to chronic infection, such as Leishmania major, the old mice showed a high proportion of pTreg cells and a low capacity to clear the infection, while Treg depletion increased Teff function in the elderly (162). Thus, the increased pTreg cells present a blockade to fighting against infection (163). b) In antitumor immunity, tumor-infiltrated Treg cells usually enhance the suppression of CD8-mediated antitumor immunity to help tumor cell survival (23, 164, 165). c) In neuronal chronic inflammation recovery, for example, in Alzheimer's disease (AD), transient and partial depletion of accumulated peripheral Foxp3 $^{+}$Treg cells caused improvement of amyloid-beta (Aβ) plaque clearance, amelioration of neuro-inflammation, and recovery in cognitive decline demonstrated in an AD mouse model (72). Mechanistically, accumulated pTreg cells suppress IFN-γ production by CD45 $^{+}$leukocytes residing at the CP, which reduced IFN-γ, thereby, failing to activate CP endothelium for the expressions of adhesion molecules, including ICAM-1 and VCAM-1. These molecules are the important mediators for the trafficking of Treg cells and reparative monocytes into the CNS for pathology resolution. Therefore, transient reduction of pTreg recues the impaired trafficking of immune cells into the inflamed CNS (72). It is not yet fully understood why more protective Treg cells than pathogenic Teff cells infiltrate via CP after transient pTreg reduction. It was reported the CNS-infiltration of Treg cells during neuroinflammation is dependent on their surface VLA-4, which is the ligand of CP expressed VCAM-1; however, CNS-infiltration of Th1 cells is independent of VLA-4 (166). Therefore, increased VCAM-1 after transient pTreg reduction can facilitate Treg trafficking more than Teff cells. Taken together, increased pan-pTreg cells in the elderly may be harmful, rather

than beneficial, for neuronal autoimmune diseases, which will be investigated by this proposed research.

Senescent T cells are accumulated with aging, and they are dysfunctional, terminally differentiated T cells (139). T cell senescence has been considered as a result of persistent lifelong antigen stimulation, particularly by chronic virus infection (167), because repeated antigen stimulation and replication can induce DNA damage of T cells, termed replicative senescence (168, 169). The dysfunction of senescent T cells is usually associated with the lost expression of CD28, and CD28 is a co-stimulatory molecule required for the second signal distinct from the TCR–peptide/MHC interaction to activate T cells and prevent anergy (170). Therefore, loss of CD28 dampens the TCR-dependent immune response of T cells (168). However, these T cells acquire chronic production of proinflammatory mediators including tumor necrosis factor (TNF) and IFN-γ (140, 171), which contributes to inflammaging conditions for age-related diseases (172-174). Loss of CD28 in $CD8^+$ T cells is an important hallmark of T cell senescence in humans, and thus $CD28^{-neg}$ $CD8^+$ T cells are the most investigated senescent T cells (175, 176). It was reported that by the age of 80, 50–60% of $CD8^+$ T cells lose their CD28 expression (177). In addition to the reduced co-stimulatory TCR signaling mediated by CD28, $CD28^{-neg}CD8^+$ T cells also present reduced TCR diversity, therefore, this T cell population presents decreased productions of IL-2 and perforin, and fails in activation and proliferation upon antigen exposure (178, 179). $CD28^{-neg}$ $CD4^+$ T cells are found in up to 70% of people over 50 years old (175). $CD28^{-neg}$ $CD4^+$ T cells is accompanied by a defect in CD154 expression, and thus they are incapable of helping B cell activation (180). However, although $CD28^{-neg}$ $CD4^+$ T cells have lost normal helper function, they produce substantial amounts of IL-2 and IFN-γ (181) and they can be autoreactive in some diseases (182, 183). Recently, age-associated cytotoxic $CD4^+$ T cells with IFN-γ and TNF-α production

have been identified in human supercentenarians (184). This cell population has a switch from TCR expression to natural killer cells receptor (NKR) expression, which supports their antigen independent cytotoxic effector functions (185). The proinflammatory profiles of accumulated senescent T cells contributes to inflammaging and thereby predispose aged individuals to neuroinflammatory diseases (186), which is an important rationale of our study on late-onset MS/EAE.

Accumulation of memory T (TM) cells with age is a sign of T cell immune aging. Within the TM population, certain numbers of cells are functionally exhausted and gain senescent characteristics. These senescent TM cells commonly secret pro-inflammatory cytokines to contribute to inflammaging (187). Accumulation of TM cells is associated with age-related thymic involution, because the maintenance of naive T cell pool in the periphery mostly relies on thymic output. However, if there are insufficient naïve T cells, peripheral clonal expansion of existing T cells by homeostatic proliferation will be enhanced, which results in oligo-clonal expansion and eventually reduces the TCR diversity (188). In addition, the turnover rates of naïve T cells by peripheral division also decline with age, especially $CD8^+$ T cells, leading to a naïve-memory imbalance (189). Aside from the intrinsic deficit, this age-related decline is also attributed to the compromised microenvironment of aged secondary lymphoid organs (190, 191). In addition to aging, infection is also an important factor to alter the microenvironment. The persistent differentiation from naïve T cells to TM cells has been conventionally considered as the life-long antigen stimulation (192); Furthermore, cumulative evidence supports the notion of "virtual memory T cells" accumulation with aging (193, 194). Virtual memory T cells are foreign antigen inexperienced and tend to be differentiated into senescent memory T cell under cytokine IL-15

stimulation (195), and aged individual have an increased IL-15 production (196) to support this differentiation.

Taken together, senescent T cell immune system with age fails to mount rapid and precise TCR dependent responses against pathogenic antigens; it instead acquires increased autoimmunity to self-tissues and an aberrant proinflammatory state. This alteration increased the risk of age-related cardiovascular, metabolic, autoimmune and neurodegenerative diseases (197).

1.7 Treg instability and plasticity

Although Treg cells are conventionally identified as the immune suppressive T cells, they can acquire instability and plasticity under inflammatory conditions. If their instability and plasticity are increased, their suppressive functions are impaired (198). Treg instability refers to the diminished Foxp3 expression with decreased suppressive function, and these Treg cells are termed “ex-Foxp3” T cells (199, 200). Treg plasticity refers to the gain of the phenotypes of other Th cell subsets, especially the proinflammatory IFN-γ producing Th1 and IL-17 producing Th17 cells, thus, they are termed Th1-like Treg cells and Th17-like Treg cells. Th-like Treg cells have a declined suppressive function on Teff cells and other inflammatory cells (201, 202). Increased instability and plasticity in Treg cells are determined in many autoimmune diseases, and these Treg cells contribute to the progression of autoimmune diseases, including type I diabetes, rheumatoid arthritis, and multiple sclerosis (203-206). Therefore, to understand the conversion conditions and effects of Treg instability and plasticity are a critical key to the understanding of T cell mediated mechanisms of MS and EAE.

Foxp3 stability is crucial for the maintenance of Treg cell function, because conditional deletion of a *Foxp3* allele in mature Treg cells lose their suppressive function (207, 208). “ex-

Foxp3" T cells derived from tTreg were found in the CNS of MOG peptide-induced EAE mice at the preclinical and peak stages of the disease, and they impaired the disease resolution (206). Given that some Tcon cells can transiently express Foxp3 and then lose Foxp3 expression to obtain Teff cell phenotypes (209, 210), the mechanism of the gain of Teff phenotypes by Treg cells is considered as cellular heterogeneity instead of reprogramming (211). Nevertheless, several lines of investigation described that the lost/diminished Foxp3 expression by Treg cells or conversion of $Foxp3^+$ Th-like Treg cells occur in response to specific signaling events, especially inflammatory environment (Fig. 4) (198). Treg plasticity can be induced by inflammatory cytokines (198). Th1-like Treg cells found in MS patients were recapitulated in vitro by culture of Treg cells isolated form healthy individuals with IL-12, a cytokine for Th1 differentiation (212). In addition, IL-12 is able to induce IFN-γ^+ Th1-like Treg cells with the expressions of Th1-related markers, including T-bet, CXCR3 and CCR5 (212, 213). Moreover, Th1-like Treg cells presented a dramatically decreased suppressive function in IFN-γ dependent manner (205). Similar to cytokine IL-12 induced Th1-like Treg conversion, IL-6 is able to induce IL-17 production by preactivated $CD4^+Foxp3^+$ Treg cells in culture (214). In addition, human peripheral blood mononuclear cells (PBMC)-derived Th17-like Treg cells were also reported to lose their suppressive capacity upon strong proinflammatory cytokine condition in the presence of IL-1β and IL-6 (215). Indeed, instability and plasticity can co-exist in Treg cells under the same inflammatory condition or inflammatory disease. For example, synovial fibroblast-derived IL-6 converted $CD4^+Foxp3^+$ Treg cells into IL-17^+ $Foxp3^{-neg}$ Th17 cells in active rheumatoid arthritis (216). Likewise, ex-Foxp3 T cells were found with IFN-γ expression in the inflamed CNS of EAE mice (206)

Given the reduced suppressive function and/or elevated proinflammatory feature, Treg instability and plasticity endows ex-Foxp3 T cells and Th1- and Th17-like Treg cells with the pathogenic roles for enhancing autoimmune diseases. The chronic inflammatory condition in the aged individuals, i.e. inflammaging, can be favorable for Treg instability and plasticity. Therefore, it remains to investigate whether Treg instability and plasticity are enhanced under inflammaging condition in the aged individuals, thereby, contributing to severe neuroinflammation and symptom in elderly MS patients and aged EAE mice.

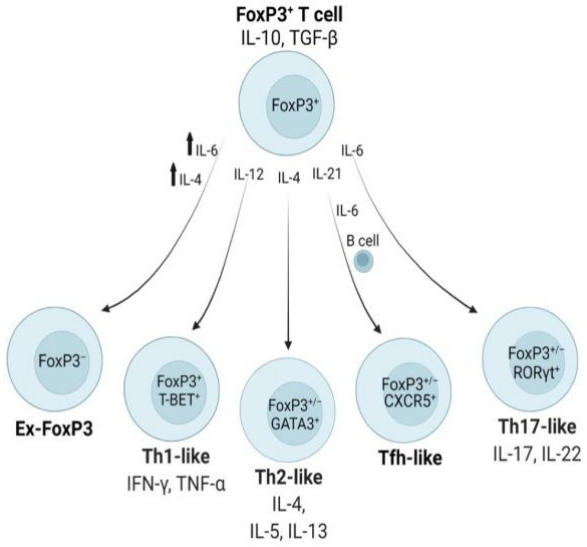


Figure 4. Cytokine microenvironments induce Treg instability and plasticity. Specific cytokine microenvironments can induce Foxp3 loss in Treg cells and repolarize Foxp3$^+$ Treg cells into various Th-like Teff cells.

1.8 Project Significance

1.8.1 Problem and Central Hypothesis

Today, people are living longer and living healthier, biomedical research for healthy aging is urgently required. However, many age-related disorders emerging in the elderly. For example, late-onset MS in the elderly has been reported, and it presented more progressive disease course than MS in young patients (127-130). In addition, the mean age of the MS population is rising (128). MS is a T cell-mediated autoimmune demyelinating disease (37). The aged T cell immune system demonstrates different characteristics from the young, exhibiting diminished normal T cell immune responses, i.e. immunosenescence (217, 218), enhanced generation of self-reactive T cells and pan-tTreg cells (33, 141, 219-221), and accumulated pan-pTreg cells in the aged periphery (142-145). Notably, although protective Treg cells are accumulated in the peripheral immune system of aged individuals, the severity of MS is increased in the aged patients (Table 1). Based on the background, we raised our central questions: Why are the MS symptoms severer in the aged patients, whereas aged patients have accumulated peripheral Treg cells? What is the underlying mechanism of this phenomena? To answer these questions a late-onset (aged) EAE mouse model, which is not yet well-established, is required for a comprehensive investigation of late-onset MS mechanism. Thus, we firstly have established a late-onset EAE model using aged C57BL/6 mice to facilitate this project.

We hypothesized that 1) Although Treg cells are protective for MS/EAE, accumulated pTreg cells in the aged individuals impairs IFN-γ involved CNS-tissue specific Treg cell trafficking via the CP into the MS/EAE inflamed CNS, which disturbs Treg distributions inside the CNS to alleviate the disease; 2) Inflammatory cytokine-rich microenvironment in the aged individuals impairs CNS-Treg suppressive function via enhancing Treg plasticity.

The hypothesis has been evaluated through three specific aims:

Specific Aim 1

To determine late-onset EAE disease course and examine pan- or MOG specific-Treg distributions in the peripheral lymphoid orangs and the inflamed CNS of aged EAE mice.

Specific Aim 2

To investigate the mechanism by which the accumulated pan-pTreg cells affect CNS-Treg distribution and contribute to late-onset EAE severity in the aged mice.

Specific Aim 3

To determine how the altered immune profiles of aged T cells are associated with neuroinflammation of late-onset EAE in the aged mice.

1.8.2 Project innovation

This project is innovative in three aspects: (1) **Animal models**: we have established a late-onset EAE mouse model using aged C57BL/6 mice for the studies of late-onset MS. (2) **Focus**: we focused on the impact of aged, accumulated pan-pTreg cells on neuronal autoimmune diseases with a late-onset EAE mouse model, and explored how the accumulated pan-pTreg cells affect disease severity and immune cell profiles inside the inflamed CNS. (3) **Concept**: we considered the aged T cell immunity not only on the immune insufficiency (immunosenescence), but also on the "immune-overloaded" problem, such as accumulated pTreg cells and hyper-self-immune

response. Thus, we raise a novel concept that age-related “immune-overloaded” is also a harmful factor to the elderly.

CHAPTER II. MATERIALS AND METHODS

Mice and animal care

C57BL/6 wild-type (WT) mice were used. Aged (18 – 20 months old) mice were ordered from aged rodent colonies of the National Institute on Aging. Control young WT mice were 2 – 3 months old. Foxp3$^{DTR/GFP}$ mice (JAX Stock No: 016958) were purchased from the Jackson laboratory and kept to the middle-aged mice in our animal facilities. Foxp3$^{DTR/GFP}$ mice express the human diphtheria toxin receptor (DTR) and *EGFP* genes from the *Foxp3* locus without disrupting expression of the endogenous *Foxp3* gene. Via i.p. injection of diphtheria toxin (DT), Foxp3^{+} Treg cells are depleted transiently. All mice were maintained under specific pathogen-free conditions in the animal facilities at the University of North Texas Health Science Center. All animal experiments were performed in compliance with protocols (IACUC-2018-0014, 2021-0020) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center, in accordance with guidelines of the National Institutes of Health.

Establishment of late-onset EAE mouse model and determination of EAE disease scores

Late-onset EAE was first induced in the common way using per mouse dosages of MOG_{35-55} peptide (WatsonBio Sciences) and pertussis toxin (PT) (List Biologicals, Cat#179A) as shown in Fig. 5. Briefly, MOG_{35-55} peptide (peptide sequence: MEVGWYRSPFSRVVHLYRNGK) was emulsified in Complete Freund′s Adjuvant (Sigma-Aldrich F5881) and given as one-time subcutaneous (s.c.) injection into the upper and lower backs of mice (200μg MOG_{35-55} peptide/mouse). The same day and second day of MOG injection, PT was intraperitoneally (i.p.) injected twice (days 0 and 1) at 200ng PT/mouse/time. Mice were monitored daily for EAE

symptoms and body weight changes. EAE scores were assigned in Table 2. Taking the impact of mouse body weight into consideration, we modified the model using body weight dependent dosages of MOG_{35-55} peptide (80μg MOG_{35-55} peptide/10g body weight) for once and PT for twice (100ng PT/10g body weight/time) (Fig. 5B).

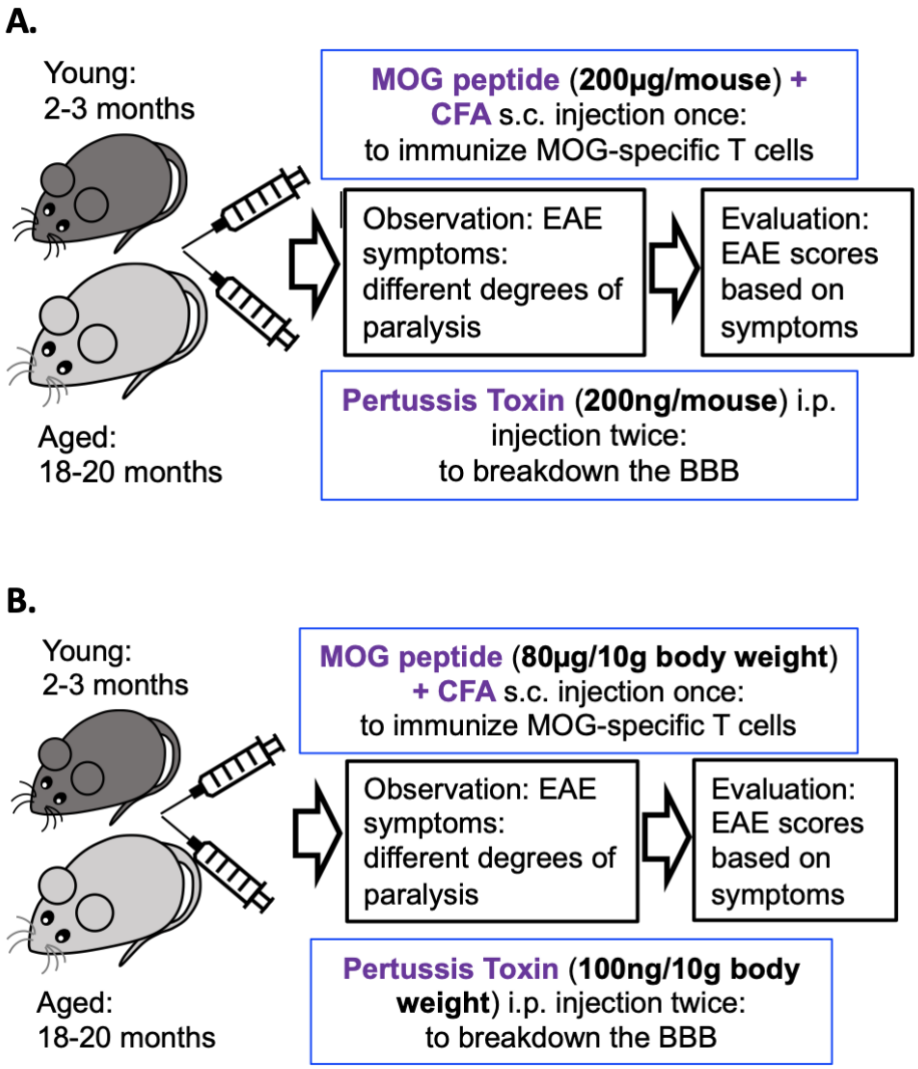


Figure. 5. Late-onset EAE mouse model and disease score observation. (A) Initial test of late-onset EAE induction model using per mouse dosages of MOG peptide and PT, followed by EAE score observation. **(B)** Modified late-onset EAE model using body weight-dependent MOG peptide and PT, followed by EAE score observation

Table 2. Mouse EAE scoring

Score	Clinic observations
0	No obvious changes in motor function compared to unimmunized mice
0.5	tail tip limpness
1.0	tail limpness
1.5	tail limpness and hind leg inhibition
2.0	partial hind leg paralysis
2.5	partial hind leg paralysis with dragging of at least one hind leg
3.0	complete paralysis of hind legs
3.5	complete hind leg paralysis and unable to right the body when placed on the side
4.0	complete hind leg and partial front leg paralysis
4.5	complete hind leg and partial front leg paralysis and mouse is not alert
5.0	spontaneously rolling in the cage or death

https://hookelabs.com/protocols/eaeAI_C57BL6.html

Transient inhibition of Foxp3 expression in pTreg cells and transient depletion of pTreg cells

For transient inhibition of Foxp3 expression in pTreg cells, a workflow is shown in Fig. 17A. Briefly, 12 days after EAE induction in young and aged WT female mice, P300i (C646; Tocris Bioscience, Cat# 4200) was i.p. injected at 8.9 mg/kg body weight per day for 5 consecutive days (5X) in one of the aged mouse groups similar to published protocol. Young and aged mice were i.p. injected with vehicle (PBS) as control groups. The dynamic changes of Treg cell frequencies and Foxp3 expression were monitored at three-time points.

For transient depletion of Foxp3 expressed pTreg cells, 12 days after EAE induction in young and middle-aged Foxp3^{DTR} mice, DT (Sigma-Aldrich, Cat#D0564) was i.p. injected at 50μg/kg body weight for one time to one of the aged Foxp3^{DTR} mouse groups. Young and another group of aged mice were injected with vehicle (PBS) as control groups.

Cell isolations from the CNS and the CP

For mononuclear cell isolation from the brains and the spinal cords, euthanized mice were transcardially perfused with 20ml of PBS. CNS tissues were minced and digested with 2mg/ml collagenase D (Roche, Cat# 11088858001) and 28U/ml DNase I (Invitrogen, Cat# 18068015) in RPMI-1640 medium at 37 °C for 45 min. Tissue was suspended in 30% percoll solution (Sigma-Aldrich, Cat# P1644) and loaded on 70% percoll solution, followed by gradient density centrifugation at 600xG for 30min at room temperature. Mononuclear cells at the interface between 30% and 70% percoll solution were collected. For cell isolation from the brain CP, CP tissues (5 mice per group) were collected from the lateral, third and fourth ventricles of the brain, then digested at 37°C for 40 min in 2mg/ml collagenase D/PBS solution with pipetting.

Tetramer-based flow cytometric assay of pan- and MOG-specific Treg and Teff cells, cytokine productions by CNS immune cells and IFN-γ producing cells from the CP

Single-cell suspensions from the lymph nodes (LNs), spleen, and the CNS of mice were stained with extracellular fluorochrome-conjugated cluster of differentiation (CD) antibodies (Biolegend, Table 3), along with APC-conjugated MOG_{38-49} $I\text{-}A^b$ (MHC-II) tetramer (NIH tetramer core, peptide sequence: GWYRSPFSRVVH) and Brilliant Violet 421-conjugated human CLIP $_{87-101}$ $I\text{-}A^b$ tetramer as a control (peptide sequence: PVSKMRMATPLLMQA). Then, cells were fixed and permeabilized using the kit (eBioscience, Cat# 00-5523-00) for intracellular staining of PE-conjugated anti-Foxp3 (eBioscience Cat# 12-5773-82) per the instruction. For intracellular staining of CNS isolated immune cells, cells were stimulated with PMA (5ng/ml), ionomycin (500ng/ml), and Protein Transport Inhibitor (0.7µl/ml, BD Biosciences. Cat# 51-2092KZ) for 5hrs at 37 °C, followed by extracellular marker and intracellular cytokine antibody staining.

Cells isolated from the CP were incubated with PMA (5ng/ml), ionomycin (500ng/ml), and Protein Transport Inhibitor as above for 5hrs, followed by extracellular staining with anti-CD45 and intracellular staining with anti-IFN-γ.

Data were acquired using BD LSR II flow cytometer and analyzed using FlowJo™ v10 software. Median florescence intensity (MFI) was defined as the "medians" of fluorescence intensities of the conjugated fluorochromes of the antibodies, based on the protocol by International Clinical Cytometry Society:

https://www.cytometry.org/web/q_view.php?id=152&filter=Analysis%20Techniques

Table 3. Antibodies used for flow cytometry staining

Reagent	fluorochrome	Catalog#	Supplier
Anti-mouse CD4	APC/Cy7	100414	Biolegend
Anti-mouse CD8	FITC	100706	Biolegend
Anti-mouse CD8	BV605	100743	Biolegend
Anti-mouse CD11b	BV711	101242	Biolegend
Anti-mouse CD25	PE/Cy7	102016	Biolegend
Anti-mouse CD45	PerCP/Cy5.5	103132	Biolegend
Anti-mouse IFN-γ	APC	505810	Biolegend
Anti-mouse Foxp3	PE	12-577-82	Thermofisher Scientific
Anti-mouse IL-17A	FITC	506903	Biolegend
Anti-mouse IL-6	PE	504504	Biolegend
Anti-mouse TNF-α	FITC	506304	Biolegend
Anti-mouse IL-10	PE/Cy7	505026	Biolegend

Analysis of transcriptome profile and TCR copy number-based T cell clonal expansion of CNS-infiltrated T cells with a single-cell RNA-seq approach

Mononuclear cells were isolated from the CNS of three young and three aged EAE mice using percoll gradient centrifugation. Cells were stained with anti-CD3-APC (Biolegend, Cat#100236) and anti-CD11b-Brilliant Violet 711 (Biolegend, Cat#101242), then $CD3^{+}CD11b^{-neg}$ T cells were sorted out on Sony SH800 Cell Sorter. Purified cells were processed to generate two cDNA libraries, including gene expression (GEX) library and TCR V(D)J library, using the 10x Genomics Chromium Single Cell 5′ GEM Library & Gel Bead Kit v2 (10x Genomics, Cat#1000287). The cDNA was amplified using the same kit. cDNA products were purified using Ampure XP beads, and the quality of cDNA was controlled using Agilent Tapestation and Qubit 4 fluorometer. TCR target enrichment was done by Chromium Single Cell Mouse TCR Amplification kit (10x Genomics, Cat #1000254). TCR V(D)J and GEX libraries were constructed by the Library Construction Kit (10x Genomics, Cat #1000190) with Dual Index Kit TT Set A. Sequencing was performed on an Illumina NovaSeq 6000 according to 10x Genomics sequencing protocol recommendations.

Fastq files (Cell Ranger, version 6.0.2, 10xGenomics provided mm10 reference genome), Cloupe file and Vloupe files were generated for the downstream analysis. The t-distributed stochastic neighbor embedding (t-SNE) plots of T cells were visualized by 10x Genomics Loupe Brower 5.0 and T cells were classified into three groups, including $CD8^{+}$ T cells, $CD4^{+}$ Teff cells and $CD4^{+}$ Treg cells. Normalized feature expressions of *Ifng* and *Il17a* were used to determine the gene expression of CNS-infiltrated $CD4^{+}$ Treg, $CD4^{+}$ Teff and $CD8^{+}$ T cell populations at the single cell level. The R packages Seurat and clusterProfiler were used for gene expression matrix generation and GO enrichment analysis. 10x Genomics Loupe VDJ Brower 4.0 was used to output

the clonotypes of $CD4^+$ Teff and $CD4^+$ Treg cells by aggregating the Cloupe file and Vloupe file of each sample. Each clone was determined by the CDR3 regions of paired TCRα and TCRβ chains. Based on TCR sequence similarity, clones with clonal size greater than 2 were defined as expanded clones, among which the most frequent 10 clones in each sample were defined as the top 10 expanded clones. All unique clones were defined as unexpanded clones.

Luxol Fast Blue (LFB) staining for demyelination assay of the spinal cords

Spinal cords were isolated from EAE mice after transcardial perfusion of PBS 28 days after EAE induction. 5μm thick paraffin sections of the spinal cord were stained with 0.1% LFB solution (Sigma-Aldrich, Cat#S3382) per a previous publication (222), with a modification by adding eosin counterstaining (Fig. 6).

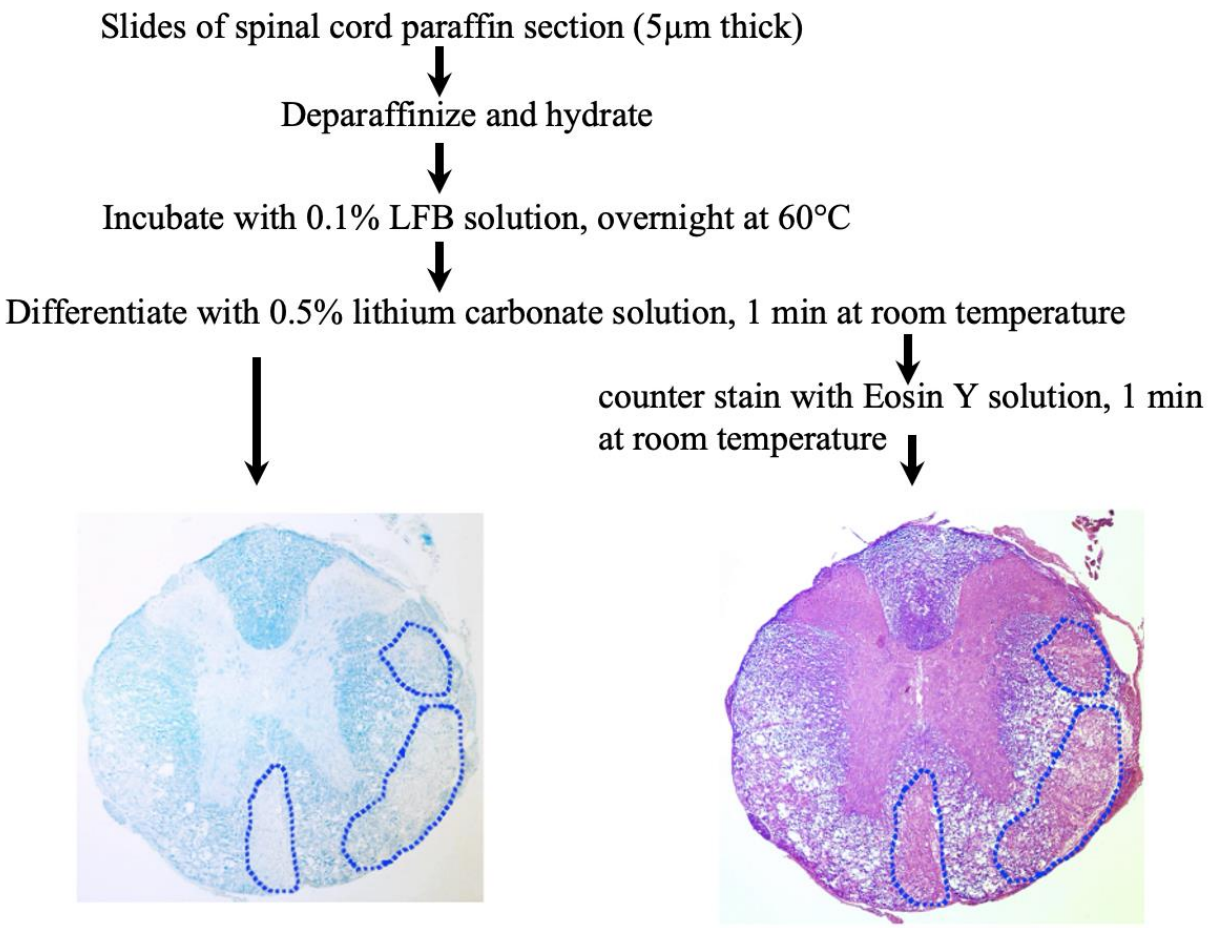


Figure 6. Luxol fast blue (LFB) staining. LFB staining* of demyelinated spinal cord with/without Eosin counterstaining.

* Yoo IH, Kim MJ, Kim J, Sung JJ, Park ST, Ahn SW. The Anti-Inflammatory Effect of Sulforaphane in Mice with Experimental Autoimmune Encephalomyelitis. J Korean Med Sci 34, e197 (2019).

Statistics

Statistical tests used to analyze each dataset of experiments are indicated in each figure legend, including the unpaired two-tailed Student's *t*-test for two groups, and one-way ANOVA for multiple groups. Data from EAE scoring were analyzed by Mann-Whitney *U* test to compare between two groups, and Kruskal-Wallis test was used to compare multiple groups followed by Dunnett's multiple comparisons post hoc test for pairwise comparisons of groups. Body weight changes over time between two groups were analyzed with two-way repeated-measures ANOVA with Geisser-Greenhouse correction. Results were considered statistically significant at values of * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Chapter III

Late-onset EAE in Aged Mice Presents Distinct Disease Characteristics, Which are Associated with Altered Treg Distribution in Peripheral Lymphoid Orangs and the Inflamed CNS

3.1 Scientific Premise and Rationale

The patients of late-onset MS are diagnosed with MS at the age of 60 or over for the first time (127-130). With the rise of the mean age of the MS population, this disease is gaining increased attention (128). Aging is a significant factor that shapes the MS disease course and correlates with increased disease comorbidities (128), such as cardiovascular comorbidities (132) and greater disability (133). Compared to young patients, elderly patients are more likely to be inflicted with progressive and severe MS including PPMS and SPMS (Table 1) (128, 133). $CD4^+$ T cells, including $CD4^+$ Teff and $CD4^+$ Treg cells, are major players and deeply involved in the initiation, development and resolution of MS and its mouse model EAE (75, 76). The balance between pathogenetic Teff cells and protective Treg cells deeply influences the outcome of the disease (223). Therefore, $CD4^+$ T cells are a focus for mechanistical investigations of MS/EAE. Of note, T cell immune system undergoes substantial changes with age, which include the following aspects: (1) age-related thymic involution leading to decreased newly generated naïve T cells (140) and increased output of autoreactive T cells (33); (2) enhanced thymic Treg generation (141), along with TCR holes created in the Treg pool (35); (3) accumulated pTreg cells in the peripheral lymphoid organs (142-145); (4) Senescent T cells involved SASP (146); (5) Increased oligo-clonal expansion of TM cells with increased functional exhaustion (147). Therefore, late-onset MS/EAE disease course and severity are assumedly remodeled by T cell

aging. It is enigmatic that aged MS patients with accumulated pTreg cells present more progressive disease courses. We hypothesized that Treg distribution in the inflamed EAE CNS is distinct from the Treg distribution in the periphery of the aged individuals. Therefore, the aim of this part of the study is to establish a late-onset EAE model using aged C57BL/6 mice, then determine the characteristics of late-onset (aged) EAE disease course and the distributions of Treg cells in the peripheral lymphoid organs and inside the inflamed CNS.

3.2 Aim-1 To determine late-onset EAE disease course and examine pan- or MOG specific-Treg distributions in the peripheral lymphoid orangs and the inflamed CNS of aged EAE mice.

3.3 Results

The course of late-onset EAE disease of aged mice exhibited its distinct characteristics

To establish a late-onset (aged) EAE mouse model, first, we immunized mice with a commonly used protocol (workflow in Fig. 7A), in which the dosage is based on per mouse by injecting 200μg MOG_{35-55} peptide and 200ng pertussis toxin (PT) to each young or aged C57BL/6 wild-type (WT) female mice (Hooke Lab etc.) (224). To our surprise, most aged mice showed a delayed onset (12 days post immunization, DPI) compared to their young counterparts (6-DPI). Eight out of thirteen (~2/3) of the aged mice showed no disease or very mild symptoms, and never reached a debility score >3.0 by 48-DPI (Fig. 7B, blue line). This observation is contradictory to the increased symptom severity and progression in aged MS patients. As the body weight is a critical factor for the dosage administration of drugs, and aged mice (30~40g) have about 50% more to double the body weights of young mice (20~25g) (web links of C57BL/6 mouse age-body weight information: See Fig. 7 legend). We believed that the unexpected EAE progression/severity

in the age mice might not be due to their low susceptibilities to MOG_{35-55} antigen, but due to the insufficient MOG_{35-55} peptide administration. Therefore, we adjusted the dosage of MOG_{35-55} peptide to 80μg/10g body weight and PT to 100ng/10g body weight (Fig. 8). By comparing young and aged female mice, we determined that aged mice had a distinct EAE onset course. We found aged mice indeed had a later EAE onset (~12-DPI), which was about 6 days later than EAE onset in young mice (as early as at 6-DPI); however, aged mice always developed severer symptoms after onset, compared to their young counterpart (blue lines in Fig. 9A, left panel: EAE scores; right panel: loss of body weight).

Given that human MS disease shows women predominance, we next compared the courses of EAE disease in aged male (Fig. 9B, green lines) and female mice (Fig. 9B, blue lines) and found the two groups had similar EAE onset days, 11~12-DPI. However, the male group had less severity with greater variation of disease symptoms, compared to the female mice. These characteristics are consistent with observations in human MS disease, in which women are the predominant population of MS patients, and also consistent with most published reports using female mice for EAE research (225). Considering homogeneity of variance, we used female mice for the rest of our studies.

Taken together, these data provided novel evidence that pathological severity and disease course of EAE disease in young and old mice are distinctively different. Therefore, this aged EAE mouse model should be used for the studies of aged human MS onset, course, and severity, which is able to provide insights into the mechanism of late-onset MS.

Different distributions of Treg cells between young and aged mice during EAE disease

Given that Treg cells play a vital protective role in the regulation of MS/EAE severity and progressive disease course (101, 105, 226, 227), we compared the distributions of pan- and MOG-specific Treg cells, along with Teff (or termed conventional T, Tcon) cells, in the periphery and the CNS between young and aged mice during EAE disease (gating strategies are shown in Fig. 10). We found similarities and differences in the distributions between young and old.

In the periphery, either in the lymph node (LN) or spleen, the percentage (%) of pan-pTreg cells in aged EAE mice was increased (Figs. 11A and 11B stripped bars in left and right panels). This is consistent with published reports regarding the accumulation of pTreg cells in aged peripheral lymphoid organs of humans and mice (142). However, % of MOG-specific pTreg cells in aged EAE mice was decreased (Figs. 11A and 11B opened bars in left and right panels). The expression of Foxp3, which is tightly associated with Treg suppressive function, was decreased in MOG-specific pTreg cells of aged EAE mice (Fig. 12). In addition, % of pan-Tcon cells was decreased (Fig. 11B, striped bars in middle panel), but % of MOG-specific Tcon cells was not changed in aged EAE mice (Fig. 11B, open bars in middle panel). The results suggest that although pan-pTreg cells are increased, MOG-specific pTreg cells are reduced. This, coupled with unchanged MOG-specific pTcon cells in the aged periphery, results in an imbalance of MOG-specific immunopathogenic and immunoregulatory T cells during aged EAE onset.

In the CNS, including the brain and spinal cord, % of both pan-Treg and MOG-specific Treg cells were decreased in aged EAE mice (Figs. 13A and 13B left and right panels). The expression of Foxp3 in MOG-specific CNS-Treg cells within the aged, inflamed CNS was decreased (Fig. 14), similar to those in their periphery (Fig. 12), indicating a decreased suppressive function. Particularly, both pan- and MOG-specific CNS-Teff cells were increased in aged EAE

mice (Fig. 13B middle panel), different from those in the periphery (Fig. 11 middle panel), suggesting both pan- and MOG-specific CNS-Treg cells are less robust in controlling CNS-Teff cell-mediated inflammation in the CNS of aged EAE mice. Interestingly, such a discrepancy between the CNS-Treg cells of young and aged mice was not observed at very early EAE onset without severe symptoms (Fig. 15), suggesting that the imbalanced CNS-Treg distribution in the aged mice is associated with EAE disease progress.

Together, the altered distribution of young and aged peripheral and CNS pan- and MOG specific-Treg cells hints the possibility that Treg cells are involved in the severity and courses of young and aged EAE diseases. These findings could help explain one of the reasons why aged mice have a late-onset but more severe EAE pathology. The pTreg accumulation may delay MS/EAE onset at the early stage of the disease. However, once the disease onsets in the aged EAE mice, the reduced pan- and MOG-specific CNS-Treg cells in the CNS results in an insufficient control of the severe and progressive disease course. In other words, it is unlikely that the accumulation of pan-pTreg cells in the periphery of aged mice can help mitigate EAE disease once it onsets in aged individuals.

3.4 Summary

Herein, we have determined the disease course and severity of late-onset (aged) EAE, and the altered distribution of peripheral and CNS pan-and MOG specific-Treg cells in aged EAE mice. EAE induction using body-weight dependent dosage of MOG_{35-55} peptide recapitulates the increased severity of clinical symptom in late-onset MS of elderly patients. Therefore, this provides a promising model for studies of late-onset MS. The distribution of Treg cells is likely associated with the severity of the disease. Although the pan-Treg cells were increased in the

peripheral lymphoid tissues of aged EAE mice, which is consistent with the fact that pTreg cells are accumulated in the aged individuals, MOG-specific Treg cells are reduced in the periphery and CNS of aged EAE mice. The loss of MOG-specific Treg cells in the aged mice can potentially reflect an impaired thymocyte agonist selection in the aged involuted thymus. In addition, not only MOG-specific Treg cells but also pan-Treg cells are reduced in the aged EAE CNS, which is also associated with the severity of the disease. This reduction is potentially attributed to an impaired Treg trafficking into the inflamed CNS for the aged EAE mice, and the underlying mechanism of disturbed Treg trafficking into the aged CNS is potentially attributed to accumulated pan-pTreg cells resident in the CNS gateways. These two points are focus of our study in the next chapter.

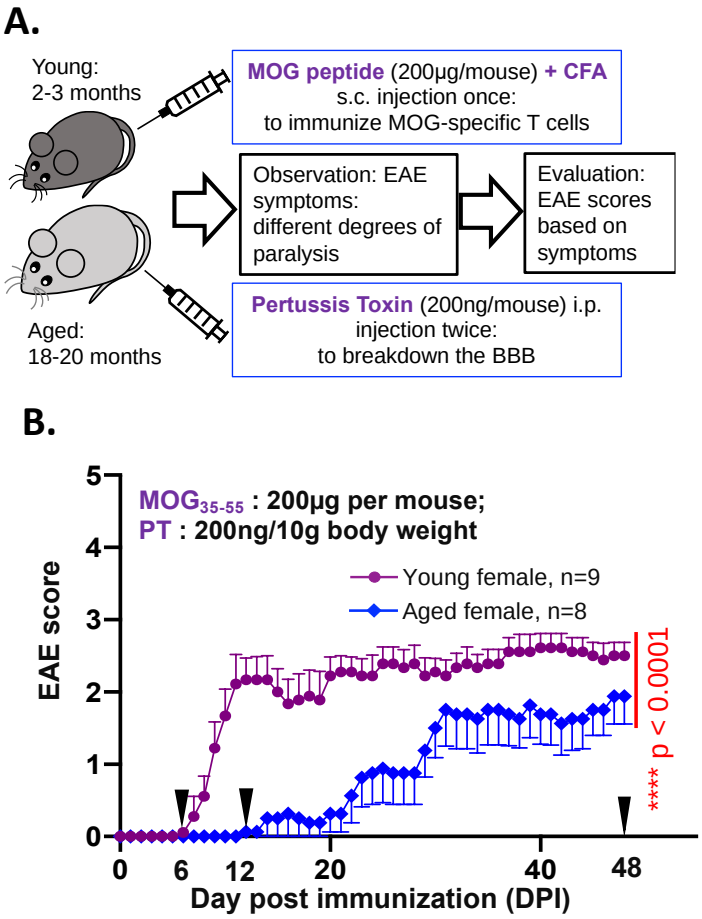


Figure 7. Establishment of late-onset (aged) mouse EAE model for aged human MS using a standard immunization protocol. (A) Workflow for EAE induction in young and aged C57BL/6 female mice using a standard immunization protocol, followed by daily EAE score evaluation. **(B)** Characteristics of EAE pathological scores in young (cherry-color line) versus aged (blue line) female EAE mice. The results indicate that with per mouse-based immunization dosage, aged mice showed a delayed onset (12 DPI) compared to the young (6-DPI). 8 out of 13 (~2/3) of the aged mice showed no disease or very mild symptoms, and never reached a debility score >3.0 by 48-DPI (blue line). This observation is contradictory to the increased symptom severity and progression in aged MS patients.
C57BL/6 mouse age-body weight information (from the Jackson laboratory):

https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664#

https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-aged-b6

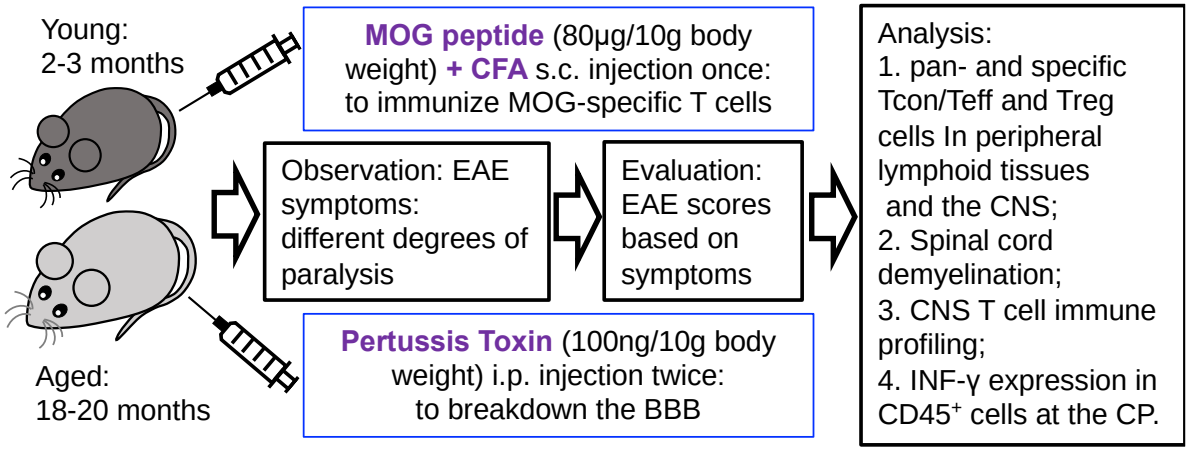


Figure 8. Workflow for EAE induction in C57BL/6 mice with body weight-dependent dosage. Each mouse was immunized with body weight dependent dosage of MOG_{35-55} peptide at 80μg/10g, along with pertussis toxin at 100ng/10g. EAE score and following tests were performed after immunization.

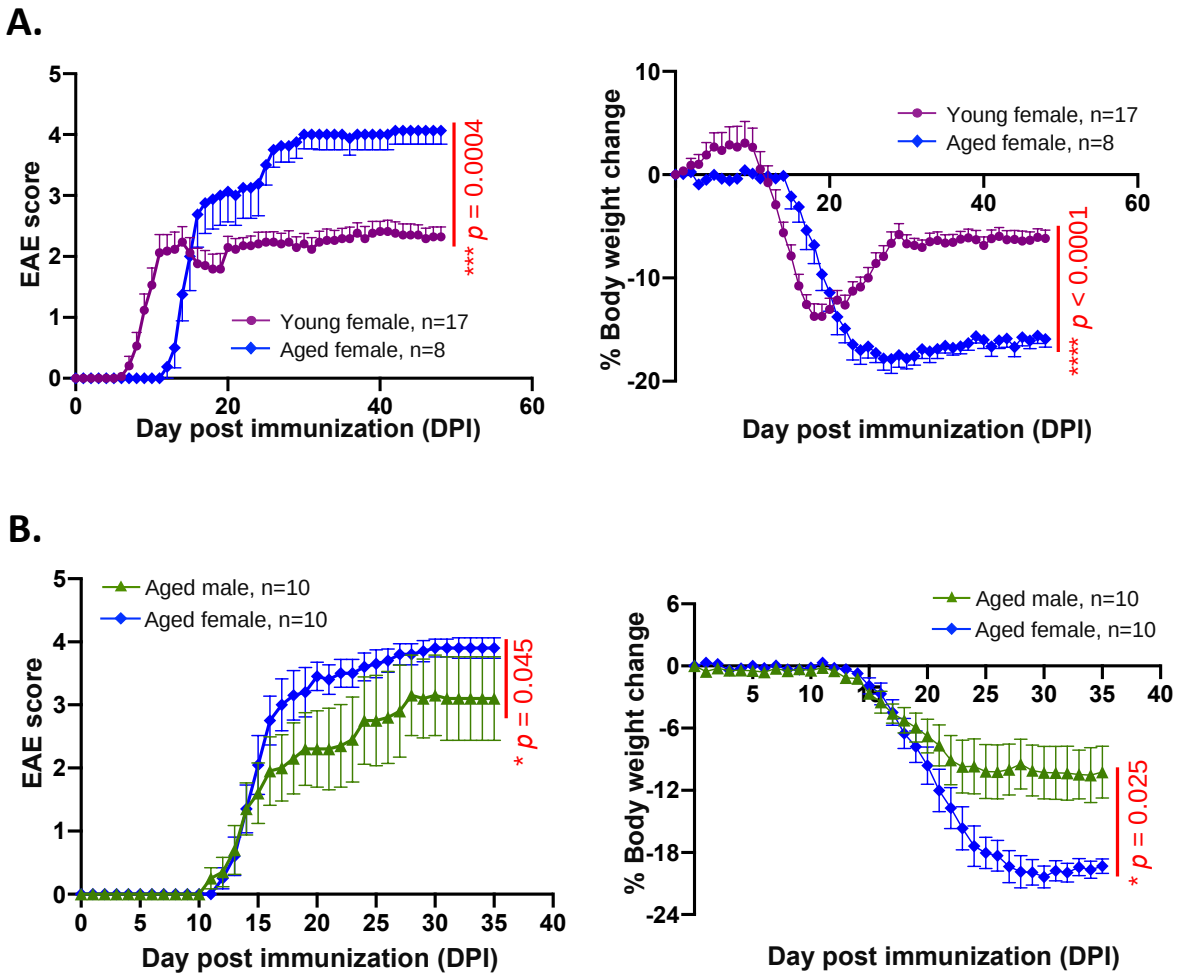


Figure 9. EAE score and body weight changes of young and aged female mice, and aged male and female mice using body weight dependent MOG$_{35-55}$ peptide dosage. (A) Characteristics of EAE pathological scores (left panel) and changes in body weights during disease (right panel) in young (cherry-color line) versus aged (blue line) female mice. **(B)** The variability of EAE pathological scores (left panel) and changes in body weights during disease (right panel) in aged male and female mice. The p-values of EAE scores were calculated by Mann-Whitney *U* test and body weight changes were calculated by two-way repeated-measures ANOVA with Geisser-Greenhouse correction, and a statistically significant difference was considered to be $p < 0.05$.

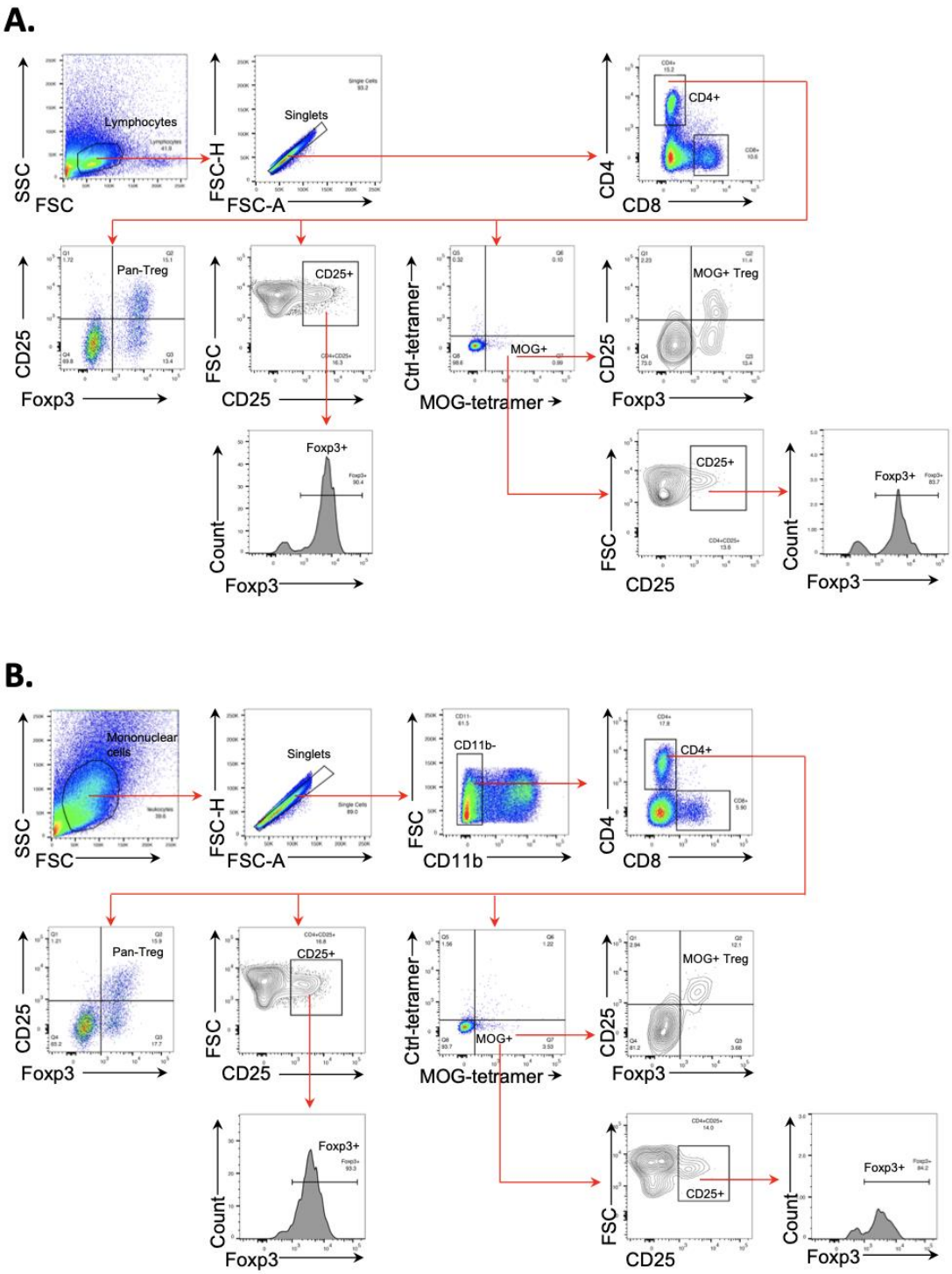


Figure 10. Flow cytometric gating strategies of T cell populations in the periphery and CNS. (A) Flow cytometric gating strategy of pan- and MOG specific- Treg and Tcon cell populations in the LNs/spleen. (B) Flow cytometric gating strategy of pan- and MOG specific- Treg and Teff cell populations in the brain/spinal cord.

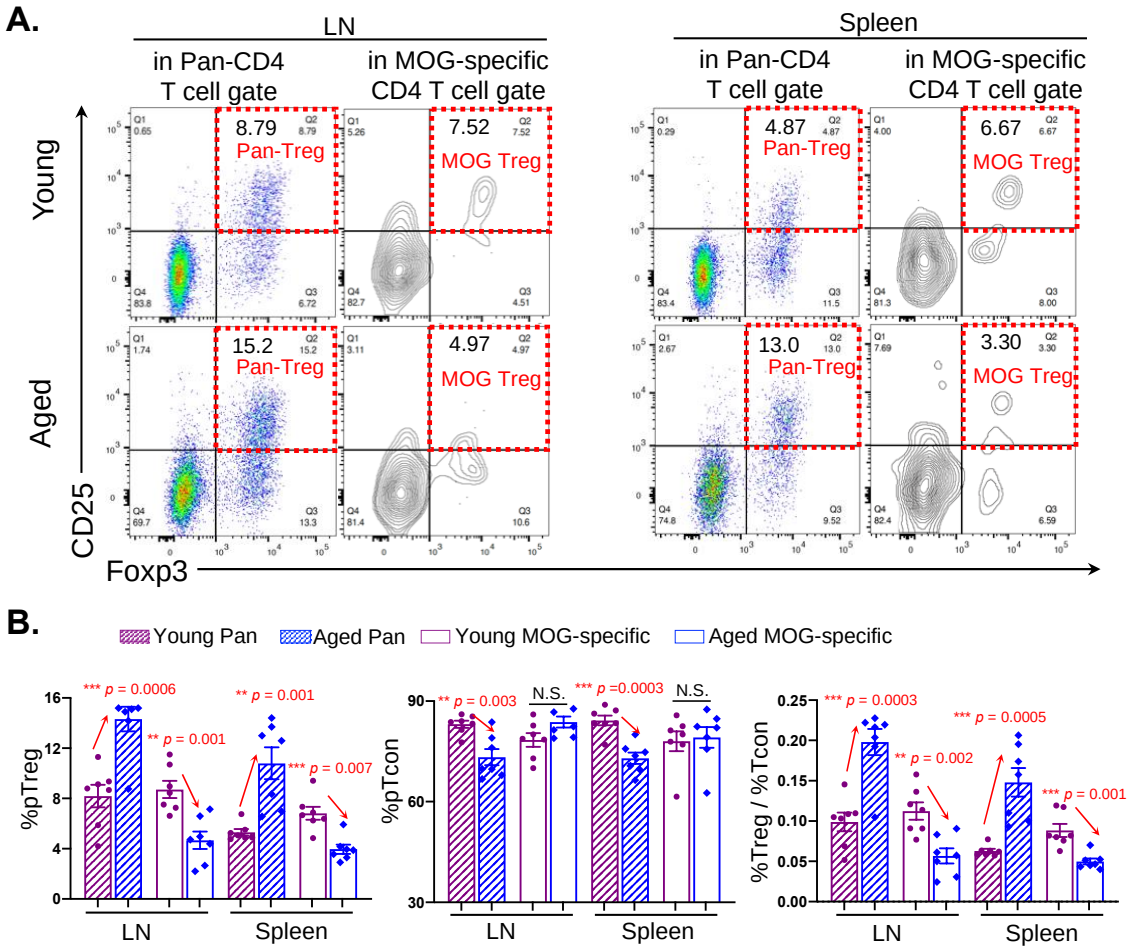


Figure 11. Distributions of pan- and MOG-specific Treg cells in the periphery of young and aged EAE mice. Mice were immunized as Fig. 8 workflow. T cells from the LNs and spleen were analyzed. **(A)** Flow cytometry gating strategies show pan- and MOG-specific Treg cells in the LNs and spleens. MOG-specific gate was determined by a dot-plot of MHC-II (I-Ab) MOG tetramer versus MHC-II (I-Ab) control tetramer (Details in Fig. 10). **(B)** Summarized results of the percentages of pan- (striped bars) and MOG-specific (open bars) Treg (left panel) and Tcon (middle panel) cells, and ratios of Treg/Tcon cells (right panel) in the LNs and spleen between young (cherry) and aged (blue) EAE mice. The *p*-values between two groups were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$, "N.S." stands for "not significant", and error bars indicate mean ± SEM.

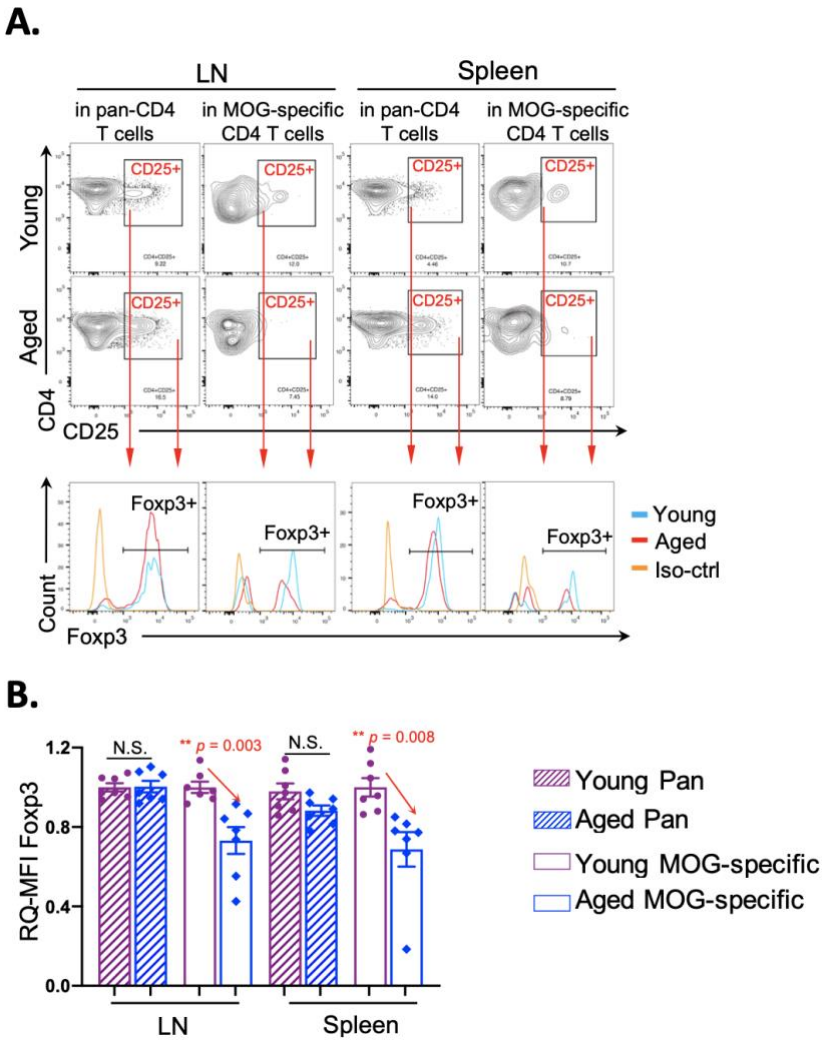


Figure 12. Foxp3 expressions of peripheral Treg cells in young and aged EAE mice. (A) Flow cytometry gating strategies show Foxp3$^+$ peaks from CD4$^+$CD25$^+$ gates of the LNs and spleens of young and aged mice. **(B)** Summarized results of the relative quantitative (RQ) median fluorescence intensity (MFI) of Foxp3 expression in pan- (striped bars) and MOG-specific (open bars) CD4$^+$CD25$^+$ populations in the LNs and spleens cord between young (cherry) and aged (blue) mice. In panels B, the *p*-values between two groups were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$, "N.S." stands for "not significant", and error bars indicate mean ± SEM.

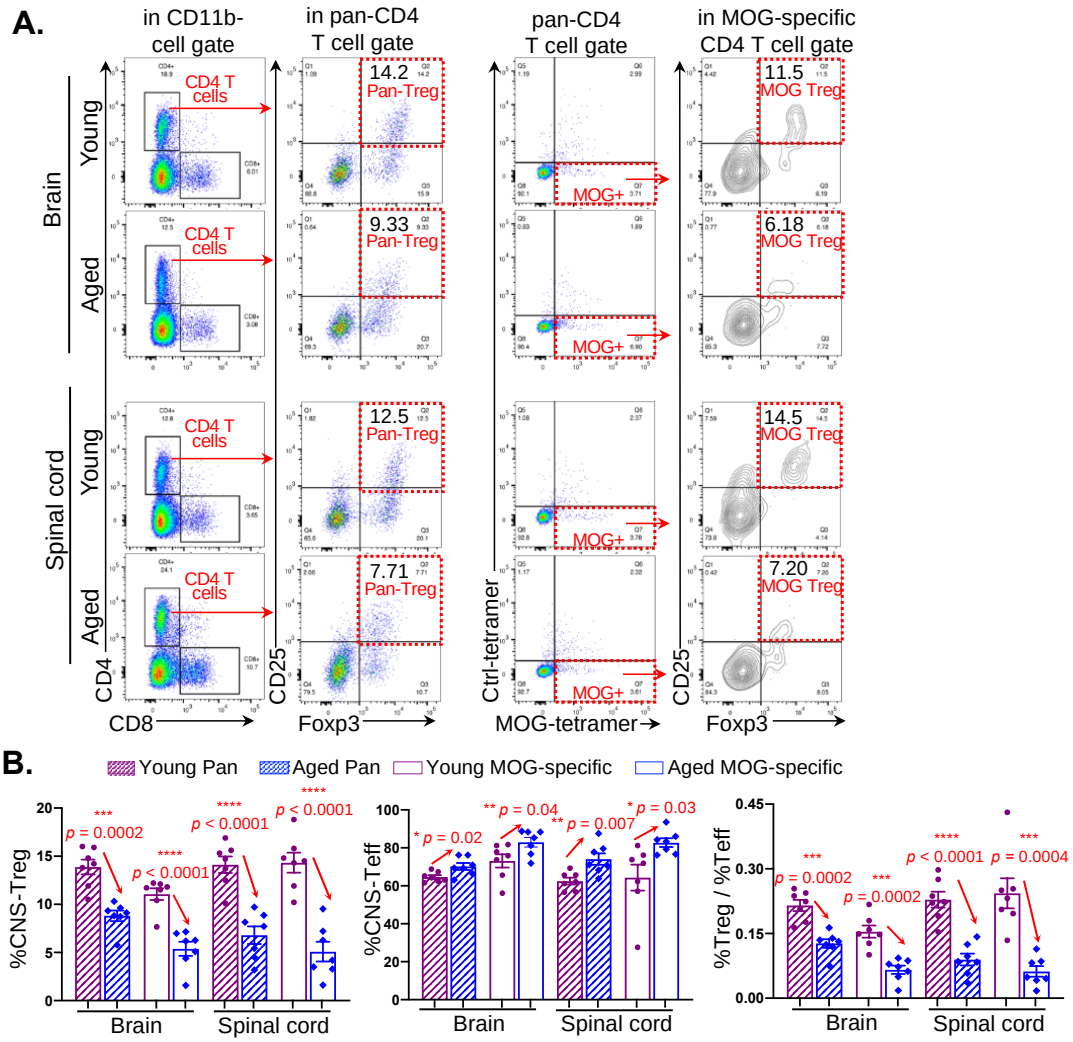


Figure 13. Distributions of pan- and MOG-specific Treg cells in the CNS of young and aged EAE mice. Mice were immunized as Fig. 8 workflow. T cells from the brain and spinal cord were analyzed. **(A)** Flow cytometry gating strategies show pan- and MOG-specific Treg cells in the LNs and spleens. MOG-specific gate was determined by a dot-plot of MHC-II (I-Ab) MOG tetramer versus MHC-II (I-Ab) control tetramer. **(B)** Summarized results of the percentages of pan- (striped bars) and MOG-specific (open bars) Treg (left panel) and Teff (middle panel) cells, and ratios of Treg/Teff cells (right panel) in the brains and spinal cords between young (cherry) and aged (blue) EAE mice. The *p*-values between two groups were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$, "N.S." stands for "not significant", and error bars indicate mean ± SEM.

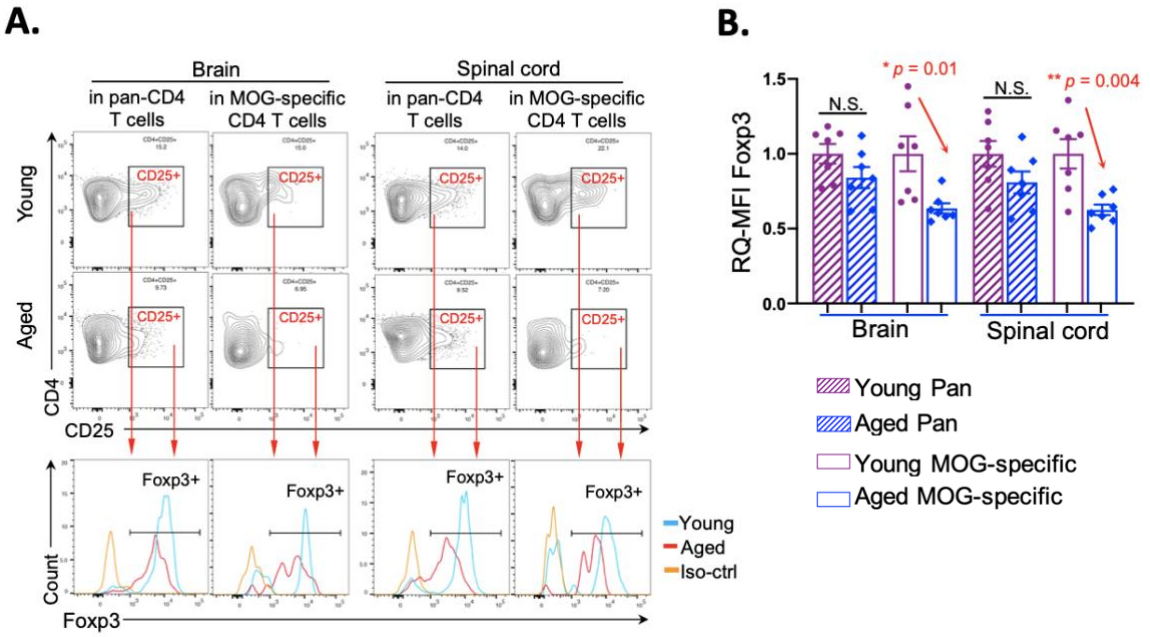


Figure 14. Foxp3 expressions of CNS-Treg cells in young and aged EAE mice. (A) Flow cytometry gating strategies show Foxp3$^+$ peaks (bottom panels) from CD4$^+$CD25$^+$ gates of the brains and spinal cords of young and aged mice. **(B)** Summarized results of the relative quantitative (RQ) median fluorescence intensity (MFI) of Foxp3 expression in pan- (striped bars) and MOG-specific (open bars) CD4$^+$CD25$^+$ populations in the brains and spinal cords between young (cherry) and aged (blue) mice. In panels B, the p-values between two groups were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$, "N.S." stands for "not significant", and error bars indicate mean ± SEM.

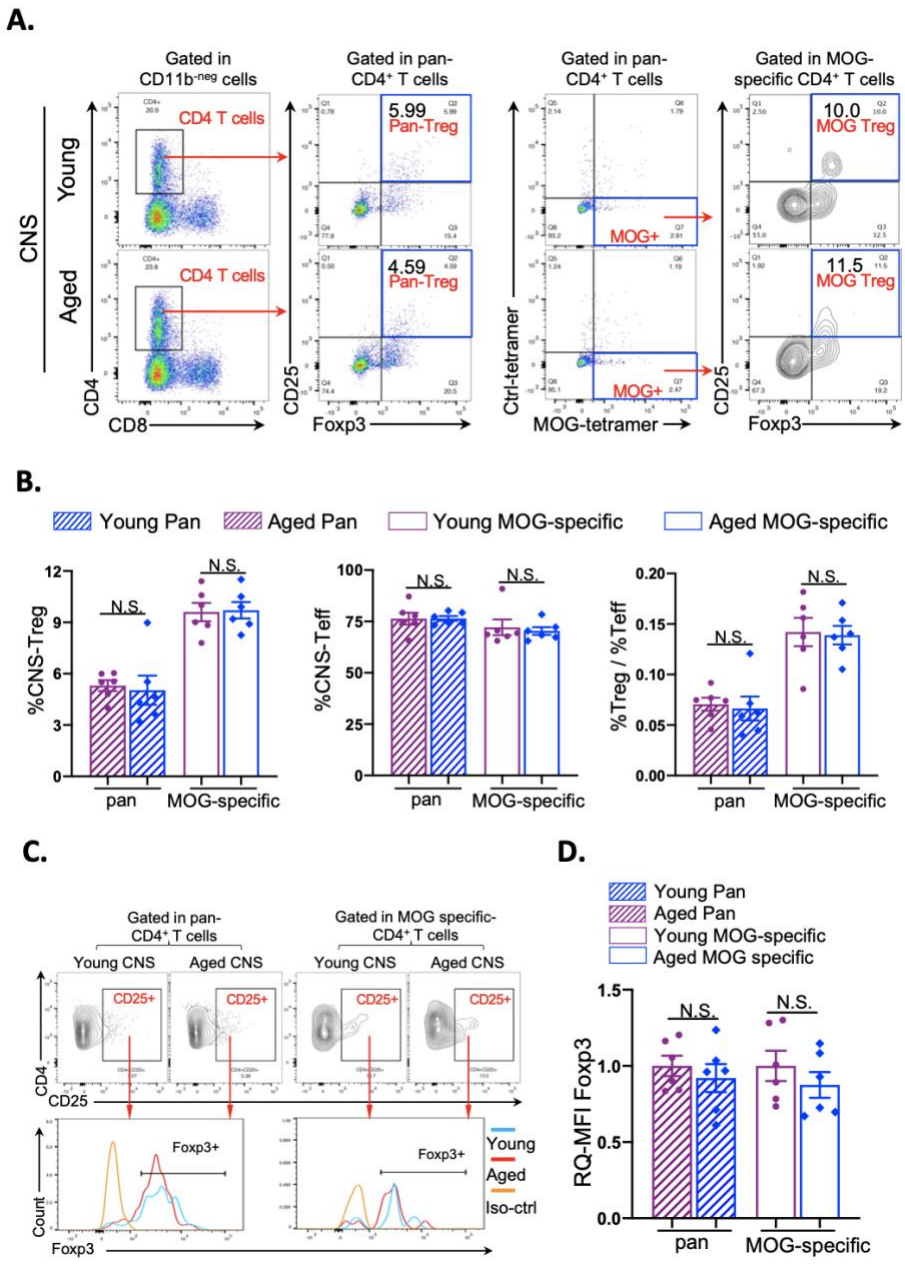


Figure 15. Distribution of pan- and MOG-specific CNS-Treg in the CNS of young and aged mice at early disease stage (8-DPI). (A) Representative flow cytometry gating strategies of pan- and MOG-specific CNS-Treg cells of young and aged mice. **(B)** Summarized results of the percentages of pan- and MOG- specific CNS-Treg cells and CNS-Teff cells, and Treg/Teff ratio of young and aged mice. **(C)** Representative flow cytometry gating strategies show Foxp3^{+} gates from CD4^{+}CD25^{+} gates of the CNS of young and aged mice. **(D)** Summarized results of the RQ-MFI of Foxp3 expression in pan-and MOG-specific CNS CD4^{+}CD25^{+} population in young and aged mice.

Chapter IV

Accumulated Peripheral Pan-pTreg cells Impair CNS-tissue Specific Treg Trafficking into the Aged EAE CNS via suppressing IFN-γ-producing leukocytes

4.1 Scientific Premise and Rationale

Reduced Treg cells in the aged EAE CNS, as we determined in the Chapter III, are potentially associated with an impeded trafficking for CNS tissue-specific Treg cell infiltration into the inflamed CNS. These CNS tissue-specific Treg cells include not only MOG-specific CNS-Treg cells, but also pan-CNS-Treg cells that recognize other CNS antigens and make a bystander effect. Because increased BBB permeability could allow the exposure of extensive CNS antigens to polyclonal T cells and various T cells can infiltrate into the inflamed CNS, the pan-Treg cells are required for suppressing inflammation. IFN-γ is a factor to increase CP permeability, therefore, this impediment of CNS tissue-specific Treg trafficking in aged EAE mice is potentially attributed to excessive pTreg cells residing at the BBB and CP, which can suppress IFN-γ-producing cells. Experiments with mouse model of AD has demonstrated that excessive Treg cells in the periphery (pTreg) was detrimental for disease resolution, because they dampened the trafficking of Treg cells and reparative monocytes into the inflamed CNS (72). The CP is a selective gateway for migration of peripheral immune cells into the CNS, and IFN-γ is a potent mediator to CP endothelium activation by upregulating its adhesion molecule expression, which allows immune cells to go through this gateway (73, 228). However, age-related accumulated pTreg cells (142, 144) can inhibit the CP resident leukocytes from IFN-γ production, thereby downregulating adhesion molecule expressions for Treg CNS trafficking through the CP gateway (72). Therefore, CP activation could be potentially inhibited by age-related excessive pTreg cells, which impair CNS-tissue specific Treg trafficking in an IFN-γ-dependent manner, resulting in compromised

neuroinflammatory resolution in MS/EAE (Fig. 16). The aim of this chapter is to verify the notion that the accumulated pTreg cells in the aged mice impede the trafficking of CNS-tissue specific Treg cells via the CP into the CNS in the late-onset EAE model. This can be confirmed by a transient pTreg inhibition or depletion to help facilitate the trafficking of Treg into the inflamed CNS, thereby alleviating late-onset EAE symptoms.

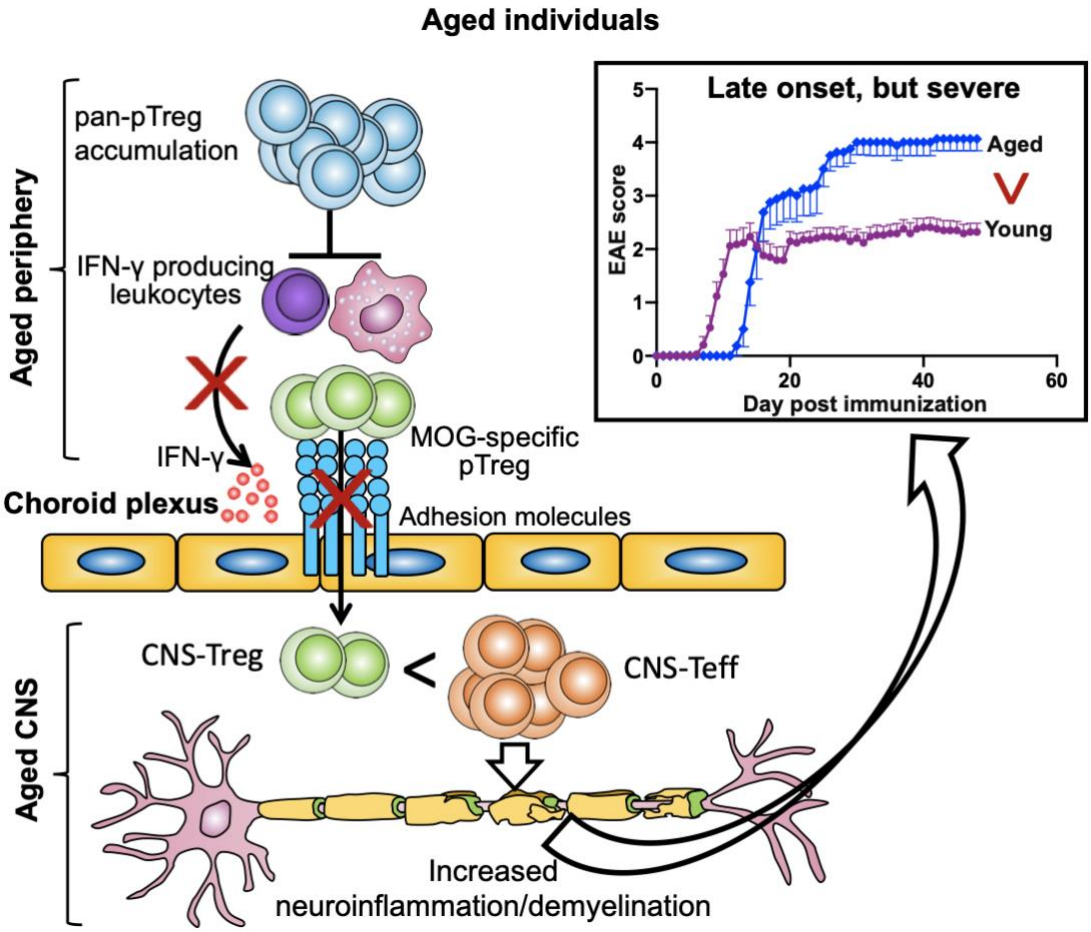


Figure 16. Aged mice have accumulated peripheral Treg cells (pTregs) with severe EAE symptoms. Accumulated pTreg cells inhibit IFN-γ mediated adhesion molecule expression at the CP. Therefore, it impairs the trafficking of CNS tissue-specific Treg cells into the inflamed EAE CNS in the age mice. Reduced CNS-Treg cells fail to effectively suppress pathogenic CNS-Teff cells and fail to alleviate neuroinflammation and demyelination, resulting in an increased symptom.

4.2 Aim-2 To investigate the mechanism by which the accumulated pan-pTreg cells affect CNS-Treg distribution and contribute to late-onset EAE severity in the aged mice.

4.3 Results

Transient inhibition or depletion of pan-pTreg cells in the aged mice mitigated EAE severity

Accumulation of pan-pTreg cells in aged individuals (142) was demonstrated to be detrimental during neurodegenerative disease AD, since transient inhibition of Foxp3 expression in the accumulated pTreg cells attenuated AD pathology (72). However, it is unknown whether this accumulation is detrimental or beneficial in late-onset (aged) MS/EAE pathology. Given that aged EAE disease accompanies with accumulated pan-pTreg cells in the periphery (Fig. 11), but reduced CNS-Treg cells in the aged, inflamed CNS (Fig.13), we hypothesized that the cellular trafficking into the CNS may be impeded by the accumulation of pTreg cells outside the CNS. Therefore, transient inhibition of Foxp3 expression in accumulated pTreg cells or transient depletion of accumulated pTreg cells in the aged mice could mitigate late-onset EAE symptoms and pathology. To confirm it, we tested this hypothesis using two mouse models. In the first model, we administrated 5 dosages of P300i to aged mice beginning on 12-DPI (Fig. 17, workflow). P300i can impair Treg suppressive activities by inhibiting Foxp3 expression without affecting Teff cell responses (229), and has been used to transiently and partially suppress pan-pTreg cells residing outside the CNS (72). We evaluated the percentages of pTreg cells (Fig. 17B) and expression level (via median florescence intensity, MFI) of Foxp3 (Fig. 17C) in the peripheral blood at three time points before and after we administrated with the P300i drug (Fig. 17A three red arrowheads indicate the tests). The results show that the pan-pTreg cells were decreased one day after the last P300i injection (17-DPI), but returned to the normal levels 12 days after the last P300i injection

(28-DPI) (Fig. 17B). This confirms that the inhibition was effective (expression of Foxp3 is drastically reduced) and transient (no more 12 days), because pTreg cells were reduced and then restored in Foxp3 expression as the pharmaceutical inhibitory effects decayed over time.

Regarding the severity of EAE disease after transient inhibition of Foxp3 in accumulated pan-pTreg cells in the aged mice, we evaluated EAE symptom (disease score) and pathology (demyelination) and found that the severity was attenuated in the aged P300i-treated group (Fig. 18A black line and Fig. 18B rightmost image). Although the treatment did not completely restore the symptom (disease score) and pathology (demyelination) of aged mice to the same level as the young group, they were significantly improved. The results provide evidence that the accumulation of aged pan-pTreg cells in the periphery does not mitigate, but rather worsen the symptom and pathology of late-onset EAE in aged mice.

To reconfirm the finding that reducing the expression of Foxp3 in accumulated pan-pTreg cells in the aged mice can rescue from EAE symptoms, we tested transient depletion of pTreg cells in the second mouse model by introducing middle-aged (12-15 months old) $Foxp3^{DTR/EGFP}$ mice (termed DTR mice) for late-onset EAE model. DTR mice have $Foxp3^{+}$ Treg cells expressing diphtheria toxin receptor (DTR), thus Treg cells can be transiently depleted by administrating diphtheria toxin (DT) (230). We intraperitoneally (i.p.) injected DT once to the middle-aged-DTR EAE mice with 50μg/kg body weight at 12-DPI. We evaluated the percentages of pTreg cells in the peripheral blood one day before the DT injection (11-DPI), one day after the DT injection (13-DPI), and 16 days after the DT injection (28-DPI). As expected, peripheral blood pTreg cells were completely depleted one day after the DT injection (Fig. 19A, red box, and 19B) and restored to the normal levels 16 days after the DT injection (28-DPI) (Fig. 19A, blue box, and 19B). As a

result, EAE symptoms were alleviated in the middle-aged-DTR mice with transient pTreg depletion (Fig. 19C).

A potential mechanism of the EAE alleviation in aged mice via transient inhibition or depletion of pan-pTreg cells is associated with reopening cellular trafficking into the inflamed CNS

We wanted to elucidate the underlying mechanism by which late-onset EAE alleviation occurred in aged mice via transient inhibition or depletion of pTreg cells. Based on a published report, when accumulated pTreg cells resided at the CP, they suppress IFN-γ-secreting cells and potentially result in hampered trafficking of monocytes and antigen-specific Treg cells into the inflamed CNS (231). Transient, rather than permanent, inhibition or depletion of the accumulated pTreg cells mitigated AD (72) due to facilitated trafficking of anti-inflammatory monocytes and Treg cells during CNS inflammation. We believe this is likely the case in late-onset MS/EAE. How do pTreg cells residing at the CP impede immune cell trafficking into the inflamed CNS? Evidence shows that Treg cells suppress IFN-γ secreting cells, while IFN-γ increases the CNS barrier permeability to facilitate the trafficking. Thus, we investigated the expression of IFN-γ in CP-adherent $CD45^+$ hematopoietic cells (mononuclear cells, etc.) and compared the proportions of CNS-infiltrated Treg and Teff cells with/without the transient inhibition of pTreg cells in the aged mice.

One day after the last treatment with P300i in aged mice (Fig. 17, the 2^{nd} test red arrow), we found that expression of IFN-γ was increased in the CP-adherent hematopoietic $CD45^+$ cells (Fig. 20A black line in the pink box, and right table). We also determined the impact on CNS-

infiltration of Treg cells after transient inhibition of pan-pTreg cells. The results show that both the percentages of both pan-Treg cells (Figs. 20B left panel and C black striped bar in left panel) and MOG-specific Treg cells (Figs. 20B right panel and C black open bar in left panel) were increased in the inflamed CNS of aged mice. The lack of change in Foxp3 expression in CNS-infiltrated Treg cells (Fig. 21) between mice with and without transient inhibition may indicate that the drug P300i does not affect CNS-infiltrated Treg cell function. In addition, increased CNS-Treg cells could either suppress CNS-Teff cells (Fig. 20C middle panel) or increase the Treg to Teff ratio in the inflamed CNS (Fig. 20C right panel). This ratio was observed to be imbalanced during late-onset EAE disease (Fig. 13B right panel). Likewise, this partially restored Treg to Teff balance in the aged EAE CNS was also observed in the middle-aged-DTR EAE mice with the transient pTreg depletion (Fig. 22). In addition, proinflammatory T cells, including both pan- and MOG-specific IFN-γ^+ Th1 cells and IL-17A$^+$ Th17 cells, were reduced CNS of DT treated middle-aged-DTR mice (Fig. 23A and B) in the. Proinflammatory cytokine produced by CD11b$^+$ myeloid immune cells were also reduced (Fig. 23C and D) in the CNS of DT treated middle-aged-DTR mice. These changes are associated with alleviated proinflammatory conditions in these middle-aged EAE CNS.

These results suggest that the potential underlying mechanism for mitigation of late-onset EAE severity via the transient inhibition or depletion of the accumulated pan-pTreg cells is through increasing INF-γ produced by the CD45$^+$ leukocytes residing at the CP, thereby, facilitating the trafficking of anti-inflammatory immune cells into the aged, inflamed CNS. This mechanism results in reduced CNS inflammation. These anti-inflammatory immune cells may include not only pan- and MOG-specific Treg cells in this study, but also other anti-inflammatory immune cells,

such as monocyte-derived anti-inflammatory cells (231). This constitutes a similar mechanism as mitigating AD pathology with this strategy (72).

4.4 Summary

Herein, we demonstrated that the reduction of pan- and MOG-specific Treg cells in the aged EAE inflamed CNS is attributed to age-related accumulation of pTreg cells, which reside in the CNS barrier. We provided counterevidence by inhibiting pTreg cells using P300i in the aged EAE mice, and depleting pTreg cells with DT in middle-aged Foxp3^{DTR} EAE mice. Both models reached the same effect, which is transient pTreg inhibition or transient pTreg depletion (Fig 17B and C). As a result of transient pTreg inhibition or depletion, EAE symptom and spinal cord demyelination in the aged EAE mice were significantly alleviated (Figs. 18 and 19). We elucidated how the transient pTreg reduction in function affected CNS-Treg trafficking via the brain CP, which is to partially reverse dampened IFN-γ expression by CP resident CD45^{+} leukocytes (Fig 20A). As the increased IFN-γ support the activation of CP endothelium for immune cell trafficking, pan- and MOG-specific Treg cells were elevated in the inflamed CNS of aged EAE mice (Fig 20B and C) and middle-aged Foxp3^{DTR} EAE mice (Fig 22 A and B). As a result, the neuroinflammation was alleviated (Fig. 23).

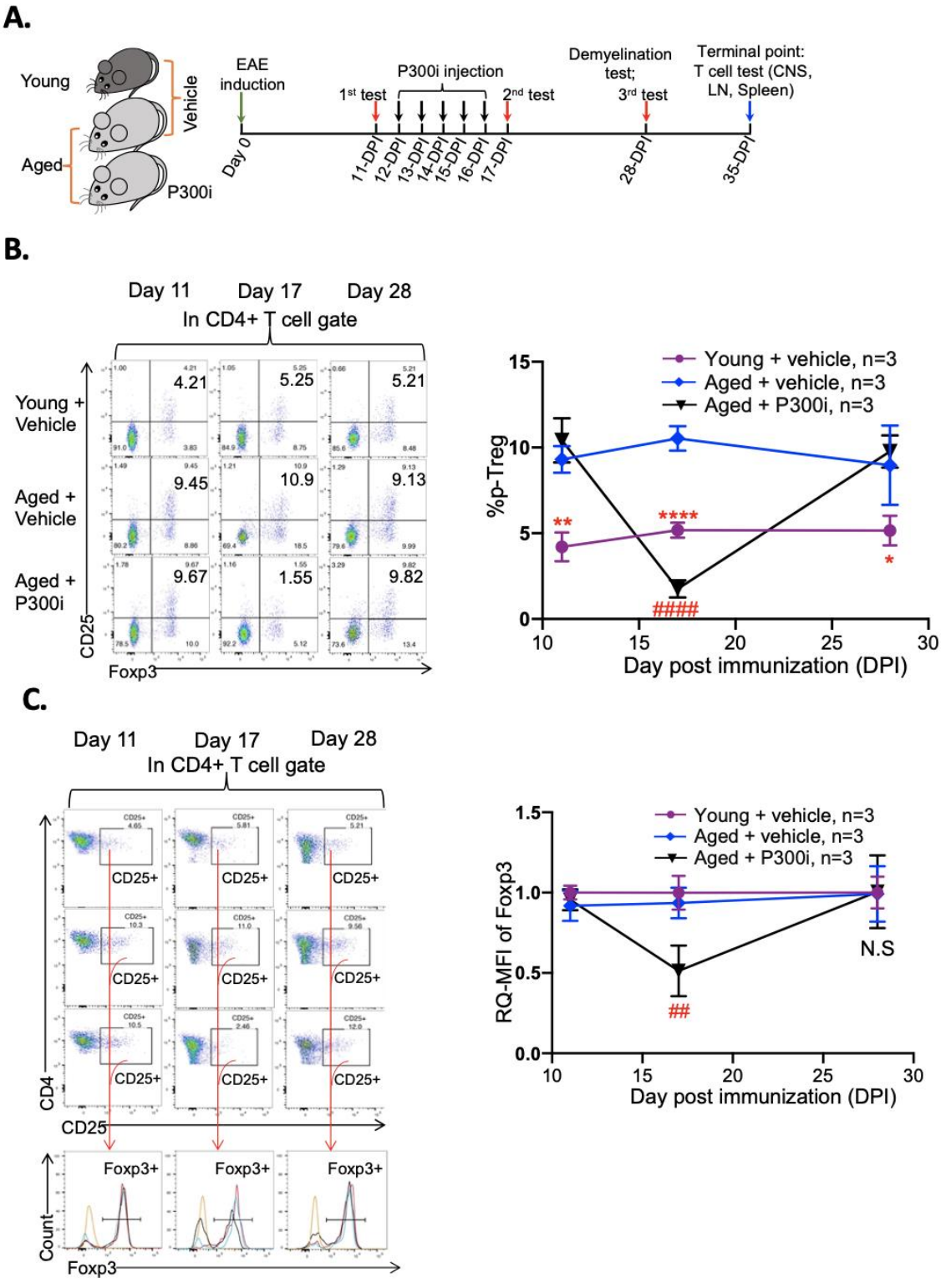


Figure 17. Transient pTreg inhibition using P300i in aged EAE mice. **(A)** Workflow of EAE induction, transient inhibition of pTreg cells, blood collection time-points (red arrowheads) for pTreg cell measurements, and demyelination assay. **(B)** Effects of transient inhibition of pTreg cells on the percentage of pTreg cells in aged EAE mice. Higher pTreg percentage in aged mice than in young mice before the first injection of P300i or vehicle (11-DPI), significantly reduced pTreg percentage after the last injection of P300i (17-DPI, black triangles/line), and restored pTreg percentage in the aged EAE mice 12 days after the last P300i treatment (28-DPI) to the control aged EAE mice (blue dots/line). **(C)** Effects of transient inhibition of pTreg cells on the Foxp3 expression of pTreg cells in aged EAE mice. Significantly reduced Foxp3 expression (MFI) in pTreg cells one day after the last injection of P300i (17-DPI, black triangles/line) in the aged EAE mice, and restored Foxp3 expression of pTreg in the aged EAE mice 12 days after the last P300i treatment (28-DPI) to the control aged EAE mice (blue dots/line). All data are expressed as mean ± SEM and were analyzed by One-way ANOVA followed by Dunnett's multiple post-hoc test. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, young +vehicle v.s. aged +vehicle; ## $p < 0.01$ and #### $p < 0.0001$, aged + vehicle v.s. aged + P300i.

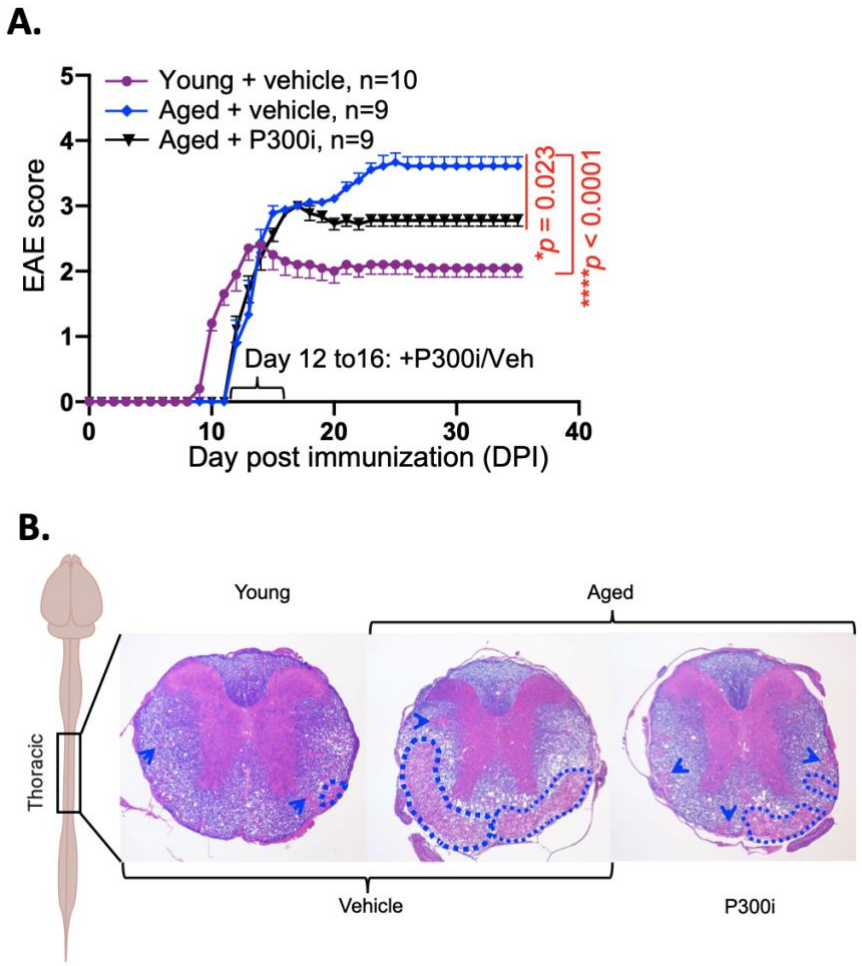


Figure 18. Alleviated EAE severity after transient pTreg inhibition in aged EAE mice. **(A)** Alleviation of the symptoms in late-onset EAE of 5x P300i-treated aged group (black triangles/line) compared to vehicle-treated control aged group (blue dots/line). **(B)** Illustration of a mouse brain and spinal cord indicating the thoracic segment of the spinal cord for LFB-eosin staining (rightmost illustration). Three representative spinal cord section images of LFB-eosin staining, showing alleviation of demyelination in the spinal cords of aged EAE mice treated with 5x P300i (right image), compared to their counterparts, the vehicle-treated age-matched control mice (middle image). The dotted outlines indicate areas of large foci of demyelination, and blue arrow heads show small foci of demyelination. This experiment was repeated three times in three mice of each group with essentially identical results.

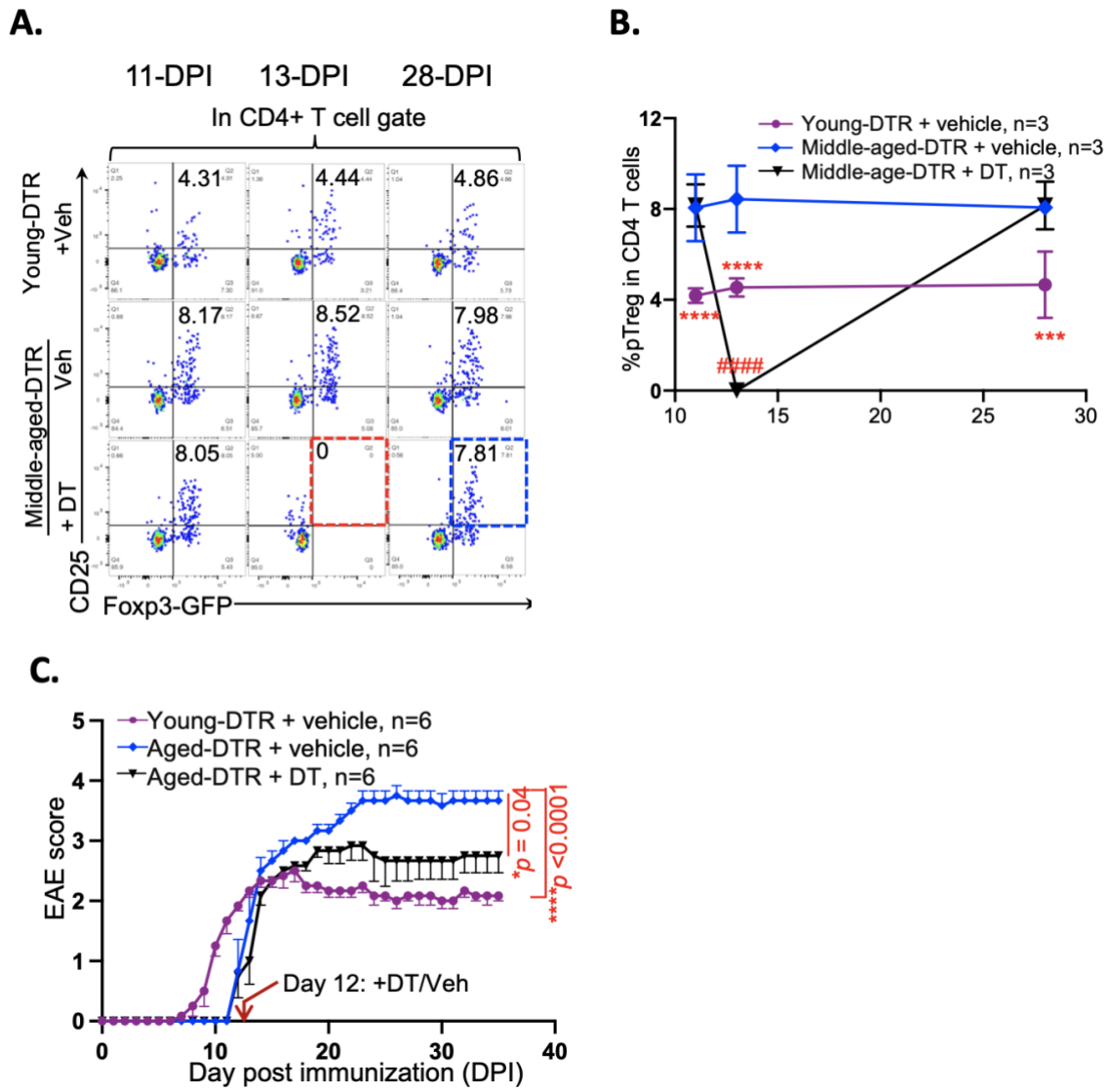


Figure 19. Time-course changes in peripheral blood pTreg cells of middle-aged FoxP3^{DTR} EAE mice with transient pTreg depletion. (A) Flow cytometry gating strategies show the frequencies of peripheral blood Treg cells in the three groups of mice 11-DPI (one day before DT treatment), 13-DPI (one day after DT treatment), and 28-DPI (16 days after DT treatment). Red box shows depletion and blue box shows recovery of pTreg cells. **(B)** Summarized results of time-course changes of pTreg frequencies in the peripheral blood of three groups of FoxP3^{DTR} EAE mice. All data are expressed as mean ± SEM and were analyzed by One-way ANOVA followed by Dunnett's multiple post-hoc test. *** $p < 0.001$ and **** $p < 0.0001$, young +vehicle v.s. middle-aged +vehicle; #### $p < 0.0001$, middle-aged + vehicle v.s. middle-aged + DT. **(C)** Alleviation of the symptoms in late-onset EAE of DT-treated middle-aged FoxP3^{DTR} EAE mice. The "n" = animal numbers.

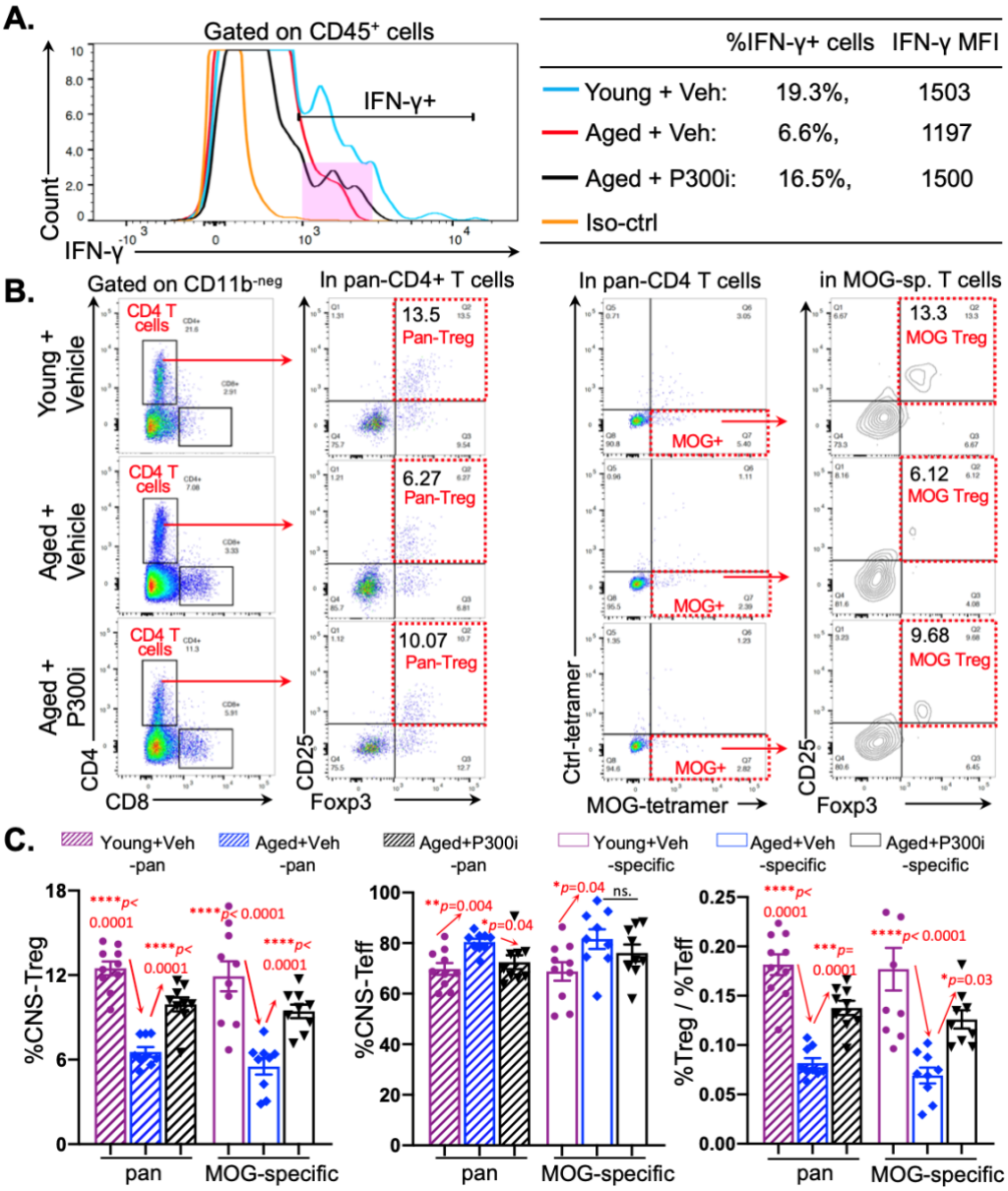


Figure 20. Alleviation of EAE in aged mice by transient inhibition of the accumulated pan-pTreg cells was potentially due to restored immune cell trafficking. The workflow is shown in Fig. 17 A. **(A)** Expression of IFN-γ in CP-residing CD45$^+$ cells were analyzed one day after the last P300i treatment (Fig. 17A, the second test red arrow). A representative histogram shows IFN-γ$^+$ CD45$^+$ cells residing at the CP isolated from the brains of young and aged EAE mice treated with P300i and vehicle, respectively. Percentages of IFN-γ$^+$ cells in CD45$^+$ cells and MFI of IFN-γ expression are listed in the table (right). **(B)** Flow cytometry gating strategies of pan-Treg and MOG-specific Treg cells in the CNS of young and aged EAE mice with P300i- or vehicle-treatment. **(C)** Summarized results of the percentages of pan- (striped bars) and MOG-specific (opened bars) CNS-Treg (left panel) and CNS-Teff (middle panel) cells, and ratios of Treg/Teff cells (right panel) in the CNS (the brain and spinal cord) of young (cherry) and aged EAE mice, treated with vehicle (blue) or P300i (black).

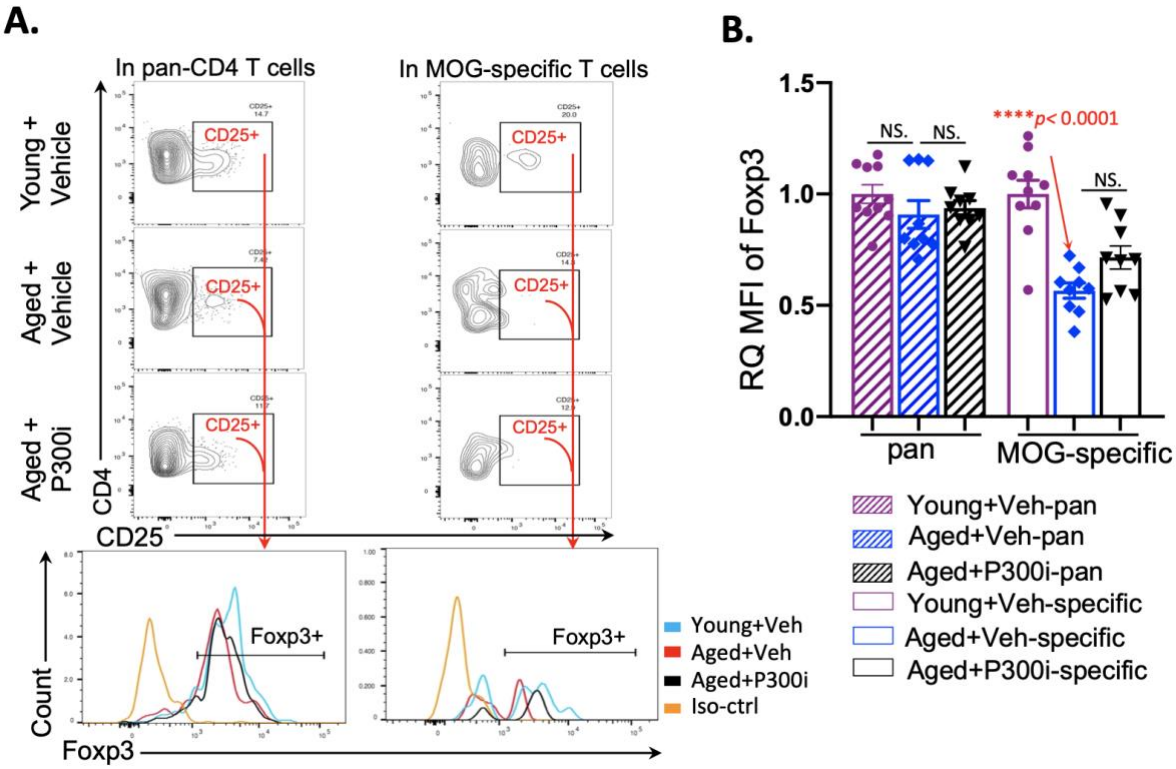


Figure 21. Foxp3 expression in CNS-Treg cells of aged EAE mice with transient pTreg inhibition. (A) Flow cytometry gating strategies show representative Foxp3$^+$ gates (bottom panels) from CD4$^+$ CD25$^+$ gates of the CNS of the three groups of EAE mice. **(B)** Summarized results of the RQ-MFI of Foxp3 expression in pan- (striped bars) and MOG-specific (open bars) CD4$^+$ CD25$^+$ population in the CNS among young (cherry) and aged EAE mice treated with vehicle (blue) or P300i (black). In panel B, each symbol represents an individual animal sample. Data are expressed as mean ± SEM. The p-values between three groups were analyzed by one-way ANOVA with a Dunnett's multiple post-hoc test, and a statistically significant difference was considered to be $p < 0.05$, "NS." stands for "not significant".

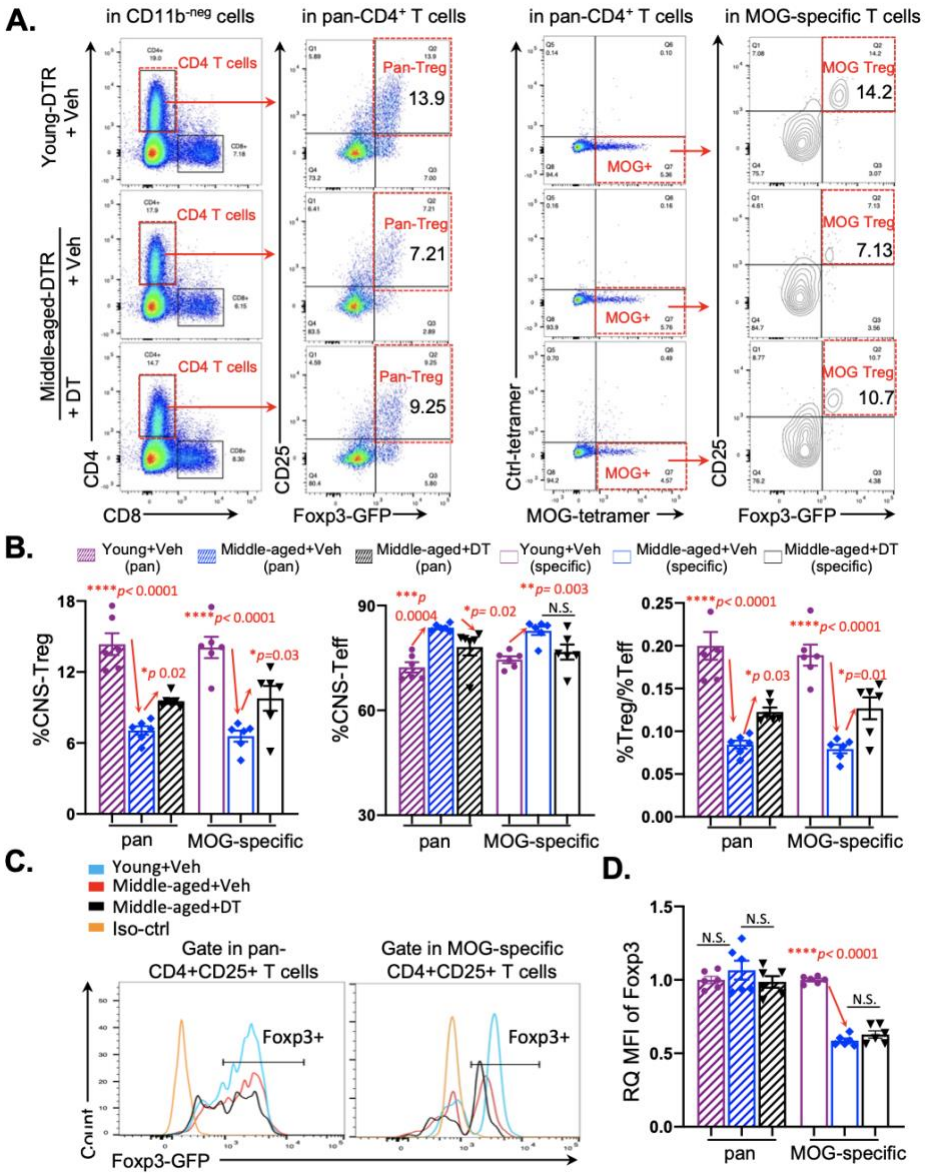


Figure 22. Transient depletion of the accumulated pTreg cells improved Treg CNS trafficking in middle-aged FoxP3^{DTR} EAE mice. (A) Flow cytometry gating strategies of pan-Treg and MOG-specific Treg cells in the CNS of young and middle-aged EAE mice with DT- or vehicle-treatment. **(B)** Summarized results of the percentages of pan- (striped bars) and MOG-specific (opened bars) CNS-Treg (left panel) and CNS-Teff (middle panel) cells, and ratios of Treg/Teff cells (right panel) in the CNS of young (cherry) and middle-aged EAE mice treated with vehicle (blue) and DT (black), respectively. **(C)** Flow cytometry gating strategies show representative Foxp3$^+$ gates from CD4$^+$ CD25$^+$ gates of the CNS of the three groups of EAE mice. **(D)** Summarized results of the RQ-MFI of Foxp3 expression in pan- (striped bars) and MOG-specific (open bars) CD4$^+$ CD25$^+$ population in the CNS of young (cherry) and middle-aged EAE mice treated with vehicle (blue) or DT (black). All data are expressed as mean ± SEM and are analyzed by One-way ANOVA followed by Dunnett's multiple post-hoc test, and a statistically significant difference was considered to be $p < 0.05$, "N.S." stands for "not significant".

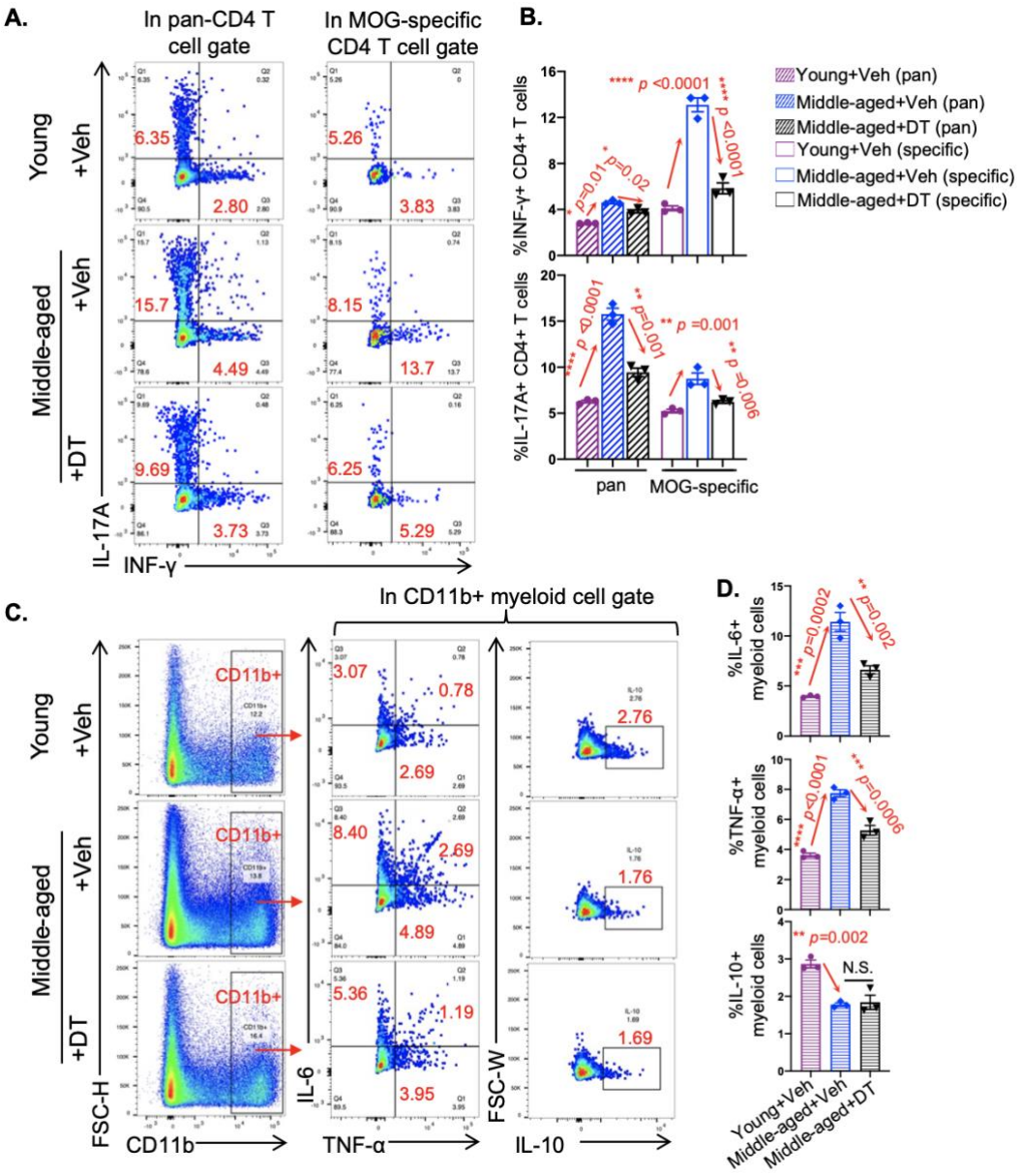


Figure 23. Inflammatory cytokine productions by CNS T cells and myeloid cells after transient depletion of pTreg cells in middle-aged Foxp3^{DTR} EAE mice. (A) Flow cytometric gating strategies of INF-γ^{+} and IL-17A^{+} pan-CD4 T cells and MOG-specific CD4 T cells in EAE CNS of young and middle-aged Foxp3^{DTR} mice with or without DT treatment. **(B)** Summarized results of the percentages of pan- and MOG-specific INF-γ^{+} and IL-17A^{+} CNS-CD4 T cells in the EAE CNS of young and middle-aged mice, treated with vehicle or with DT. **(C)** Flow cytometric gating strategies of IL-6^{+}, TNF-α^{+} and IL-10^{+} myeloid immune cells in the EAE CNS of young and middle-aged Foxp3^{DTR} mice with or without DT treatment. **(D)** Summarized results of the percentages of IL-6^{+}, TNF-α^{+} and IL-10^{+} myeloid immune cells in the EAE CNS of young and middle-aged mice treated with vehicle or with DT. The results display that transient depletion of accumulated pTreg cells in middle-aged mice results in reduction of proinflammatory cytokines in the inflamed CNS.

Chapter V

Enhanced Treg Plasticity in the Inflammatory Condition Impairs the Suppressive Function on Teff cells in the Aged EAE CNS

5.1 Scientific Premise and Rationale

Aside from the unfavorable inhibitory effect due to the increased pTreg population on CP activation, the immune regulatory profile of CNS-infiltrated Treg cells may be altered at the single cell level. This alteration also contributes to the outcome of an inflammatory disease, as reduced Treg suppressive function was found in autoimmune diseases (232). One of the prominent functional alterations of Treg cells is Treg plasticity, which refers to gain of the phenotype of other Teff cells, and reduced suppressive function on Teff cells and other inflammatory cells (201, 202). IFN-γ-producing Th1-like Treg cells and IL-17-producing Th17-like Treg cells are two mostly reported populations in autoimmune diseases for understanding the detrimental effect of Treg plasticity. IFN-γ and IL-17 are two important pathogenic cytokines involved in for MS pathogenesis (203-205). In addition, Treg plasticity-resulted Th1 or Th17 polarization impairs the suppressive function of Treg cells against Teff cells (205, 215), thereby enhancing the inflammation in autoimmune disease.

One of the important etiologies of Treg plasticity is the inflammatory cytokine-rich environment in autoimmune diseases (198). Therefore, the chronic inflammatory condition existing in the aged individuals provides a favorable condition to promote Treg plasticity. Taking this into account, how the plasticity of Treg cells affects neuroinflammation in the CNS of late-onset EAE will be explored in this chapter.

5.2 Aim-3 To determine how the altered immune profiles of aged T cells are associated with neuroinflammation of late-onset EAE in the aged mice.

5.3 Results

CNS-infiltrated Treg cells in late-onset EAE of aged mice exhibited dysfunctional molecular profiles

Treg cells can possess relatively unstable features (233, 234), including down-regulation of Foxp3 expression (200, 206) as confirmed in our late-onset (aged) EAE mouse model (Fig. 12B and 14B), and co-expression of *Ifng* or *Il17a* toward Th1-like or Th17-like plastic conversion (203, 235) upon autoimmune stimulation (200, 206). This leads to functional plasticity, resulting in increased pathology and reduced suppressive capacity (200, 235). We believe that this unstable phenotype is likely more prominent in the aged inflammatory microenvironment. To evaluate these function-related molecular profiles, we analyzed the expression of *Ifng* and *Il17a* genes in CNS-infiltrated Treg cells of EAE mice at the single-cell level (Fig. 24A, workflow). We compared $CD4^+Foxp3^+$ CNS-Treg cells in the young and aged inflamed CNS (Fig. 24B) during EAE disease and found that expression of *Ifng* and *Il17a* was indeed increased in the CNS-infiltrated aged Treg cells (Fig. 24C). In addition, we noticed that CNS-$CD8^+$ T cells showed increased *Ifng* and *Il17a* expression levels in the aged CNS with EAE disease (Fig. 24D). These CNS-$CD8^+$ T cells are activated and pathogenic due to their expression of *Ifng* and *Il17a*, which potentially lead to autoimmune encephalomyelitis (114, 236). In addition, in the typical EAE pathogenic $CD4^+$ T cell subsets Th1 and Th17 cells, the expressions of *Ifng* and *Il17a* were increased in the aged, inflamed CNS (Fig. 24E). Gene Ontology (GO) enrichment analysis of CNS-infiltrated $CD4^+$ T cells in aged

vs. young EAE mice showed that expressions of genes involved in T cell activation and proliferation pathways were up-regulated in aged CNS-Teff cells (Fig. 25A), but expressions of those genes were not up-regulated in aged CNS-Treg cells (Fig. 25B). The results imply that aged Treg cells, which have infiltrated into the CNS after EAE onset, could not have sufficient capacity to suppress enhanced CNS-Teff cell activation in inducing neuronal inflammation.

Clonal expansion of CNS-infiltrated Teff cells in late-onset EAE in aged mice was increased

To further evaluate whether CNS-infiltrated aged Treg cells have reduced suppressive capacity against CNS-Teff cells, an assessment of CNS-Teff cell clonal expansion in the EAE CNS can shed certain light. We therefore analyzed clonal expansion in the CNS-infiltrated Teff cell pool along with the Treg cell pool based on TCR sequence similarity via TCR$\alpha\beta$ immune profile analysis with the ingle-cell RNA sequencing (scRNA-Seq) approach (Fig. 26). The suppressive capacity of CNS-Treg cells can be reflected by their effect on suppression of clonal expansion of CNS-Teff cells, because reduced suppressive capacity of CNS-Treg cells will result in increased clonal expansion of CNS-Teff cells in the same compartment. The results showed that aged CNS-Teff cells had greater clonal expansion, compared to their young counterpart (Fig. 26A top pies and Fig. 27 left two columns), and the expanded CNS-Teff clones (both total expanded clones and top 10 expanded clones) occupied a higher proportion of the CNS-Teff pool (Fig. 26B left panels) in the aged, inflamed CNS. However, although Treg cells could proliferate in vivo (237), aged CNS-Treg clones showed a similar expansion as in their young counterpart (Fig. 26A bottom pies, 26B right panels, and Fig. 27 right two columns). Together, activation of aged CNS-Teff cells is increased in the aged, inflamed CNS during EAE disease, which is potentially due to insufficient suppression by CNS-Treg cells in the same compartment. The results also indicate that

the expansion capacity of CNS-infiltrated Treg cells in aged EAE mice is not reduced compared to their young counterparts.

5.4 Summary

Herein, we determined a mechanism of increased pathology of late-onset EAE, which is associated with the increased Treg plasticity in the aged, inflamed EAE CNS. Our results from scRNA-seq analysis of CNS-$CD3^+$ T cells of young and aged EAE mice revealed the molecular profiles of $CD4^+$ Treg cells, $CD4^+$ Teff cells and $CD8^+$ T cells (Fig. 24A and B). Gene expressions of *Ifng* and *Il17a* were increased in $CD4^+$ Treg cells in the CNS of aged EAE mice (Fig. 24C), indicating the increased Treg plasticity and decreased suppressive function. As a result of impaired Treg suppression, gene expressions of *Ifng* and *Il17a* in $CD8^+$ T cells and $CD4^+$ Teff cells were also upregulated (Fig 24D and E), which suggests enhanced neuroinflammation in the aged EAE mice. This impaired Treg suppression was further supported by (1) GO analysis, which revealed the enriched expression in the T cell activation and proliferation in the aged $CD4^+$ Teff cells (Fig 25A), and (2) clonal expansion assay which displayed increased clonal expansion of $CD4^+$ Teff cells (Fig. 26) in the CNS of aged EAE CNS. In summary, Treg cells in the inflamed CNS of aged EAE mice have increased plasticity, which leads to reduced suppression on CNS-Teff cell-induced inflammation, thereby, enhancing proinflammatory effects.

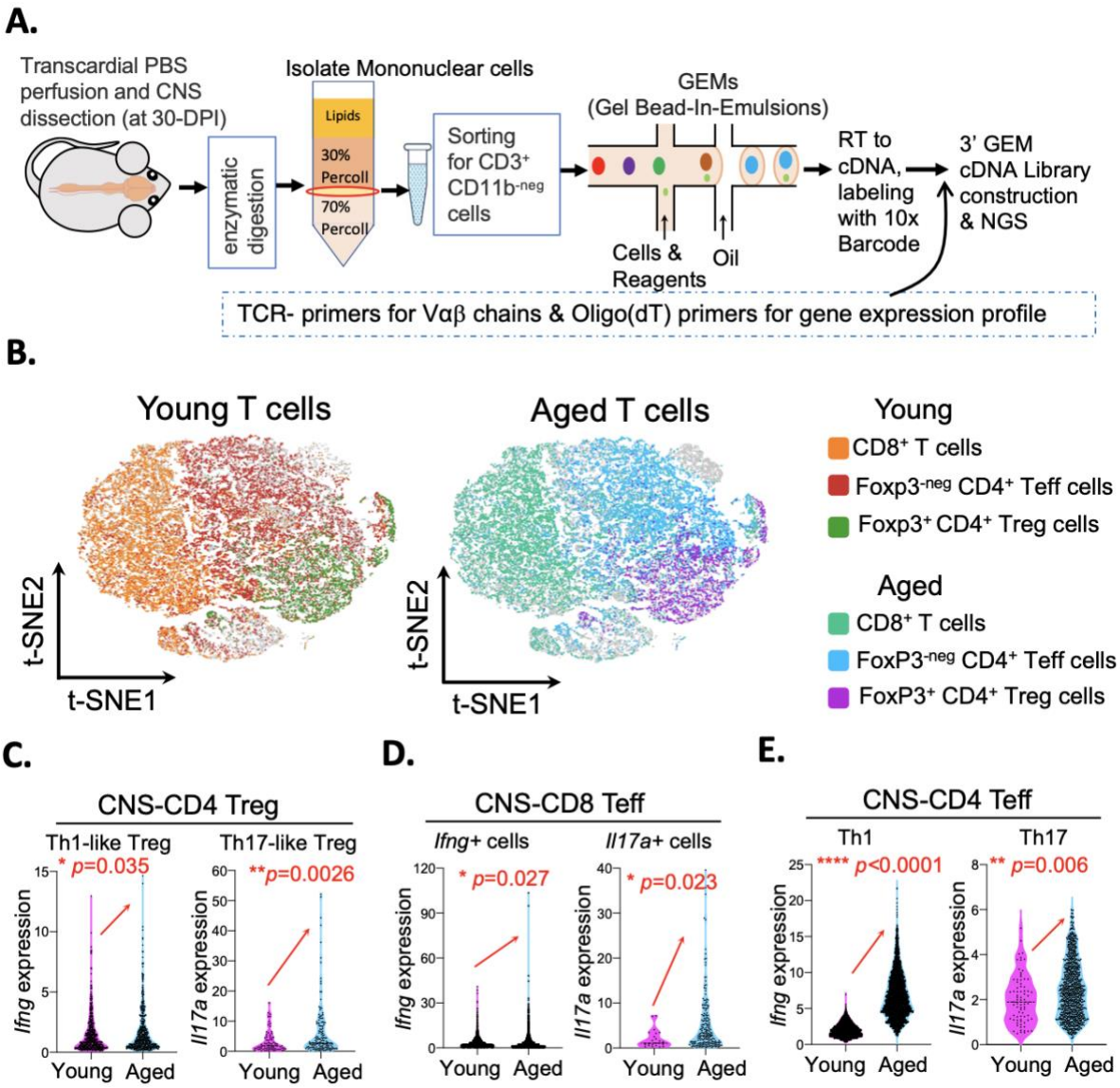


Figure 24. Function-associated molecular profile analysis for CNS-Treg plasticity and pro-inflammatory CNS-CD8$^+$ T cells and CNS-CD4$^+$ Teff cells of EAE mice. Mice were immunized as in Fig. 8 workflow. **(A)** Workflow of sc-RNA-seq for CNS infiltrated T cells. T cells in the CNS (the brain and spinal cord) were isolated via gradient centrifugation and sorted for CD3$^+$ CD11b^{-neg} cells by flow cytometry 30 days after immunization. Then, single cells were captured and emulsified with specific gel beads for reverse transcription and construction of cDNA library for high-throughput sequencing. **(B)** The pattern of young (left t-SNE plot) and aged (right t-SNE plot) T cells from the CNS of three young and three aged EAE mice. **(C-E)** Normalized expressions of *Ifng* (left panel) and *Il17a* (right panel) genes in single Th1- and Th17-like Treg (CD4$^+$FoxP3$^+$) cells (C), CD8$^+$ T cells (D) and CD4$^+$ Teff (CD4$^+$ FoxP3^{-neg}) cells (E) of the young (cherry) and aged (blue) EAE CNS. Data in panels were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$. Each symbol represents a single cell.

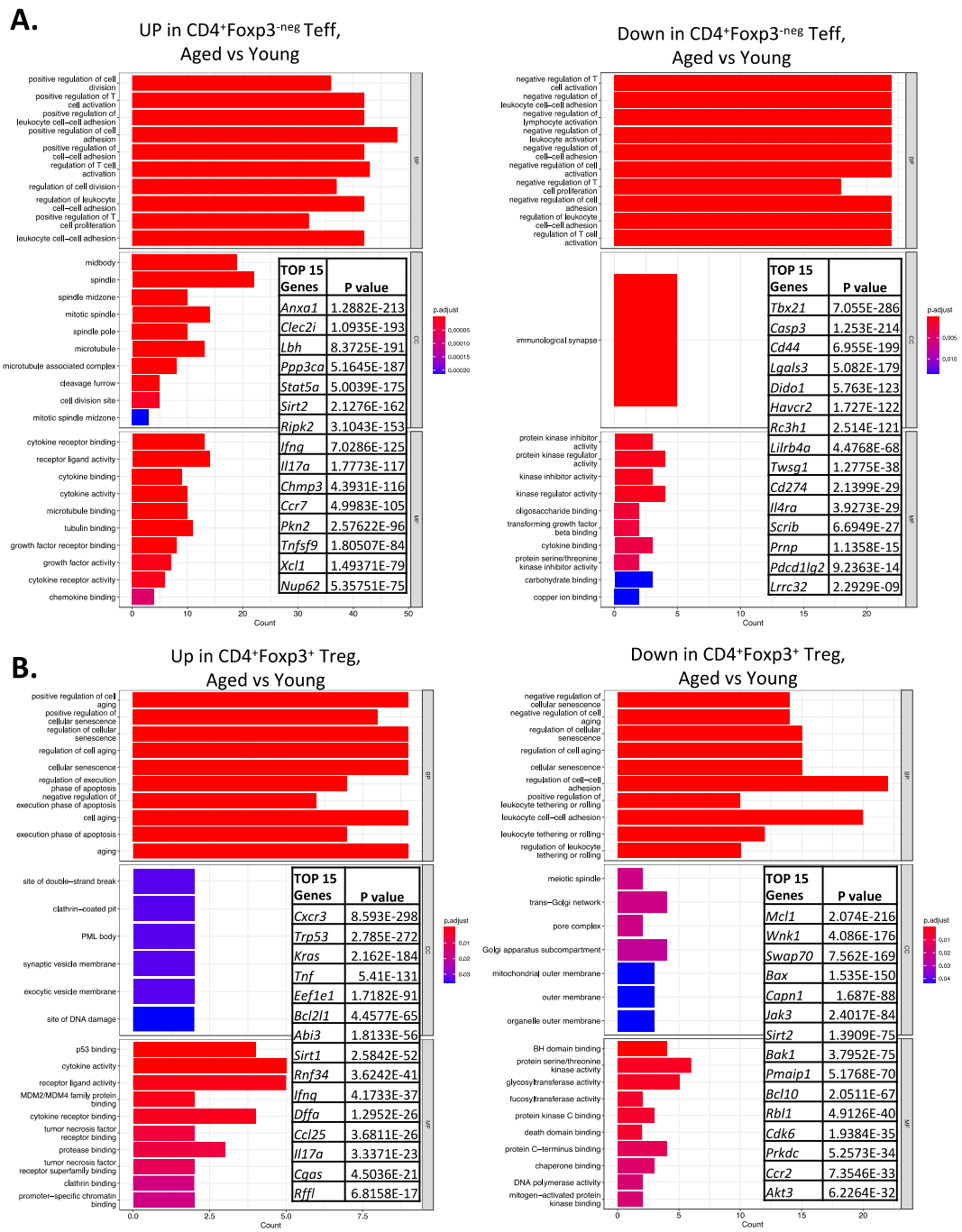


Figure 25. GO enrichment analysis of up-regulated and down-regulated gene sets in aged CD4⁺ CNS-Teff cells and CD4⁺ CNS-Treg cells. (A) GO enrichment analysis of up-regulated (left panel) and down-regulated (right panel) gene enriched pathways in aged CD4⁺Foxp3^{-neg} CNS-Teff cells with the top 15 significantly upregulated and downregulated genes (inserted boxes in the left and right panels) of aged CD4⁺Foxp3^{-neg} CNS-Teff cells. **(B)** Same analysis as in (A) of aged CD4⁺Foxp3⁺ CNS-Treg cells. The results imply that aged Treg cells, which have infiltrated into the CNS after EAE onset, could have reduced capacity to suppress CNS-Teff cell-induced neuronal inflammation.

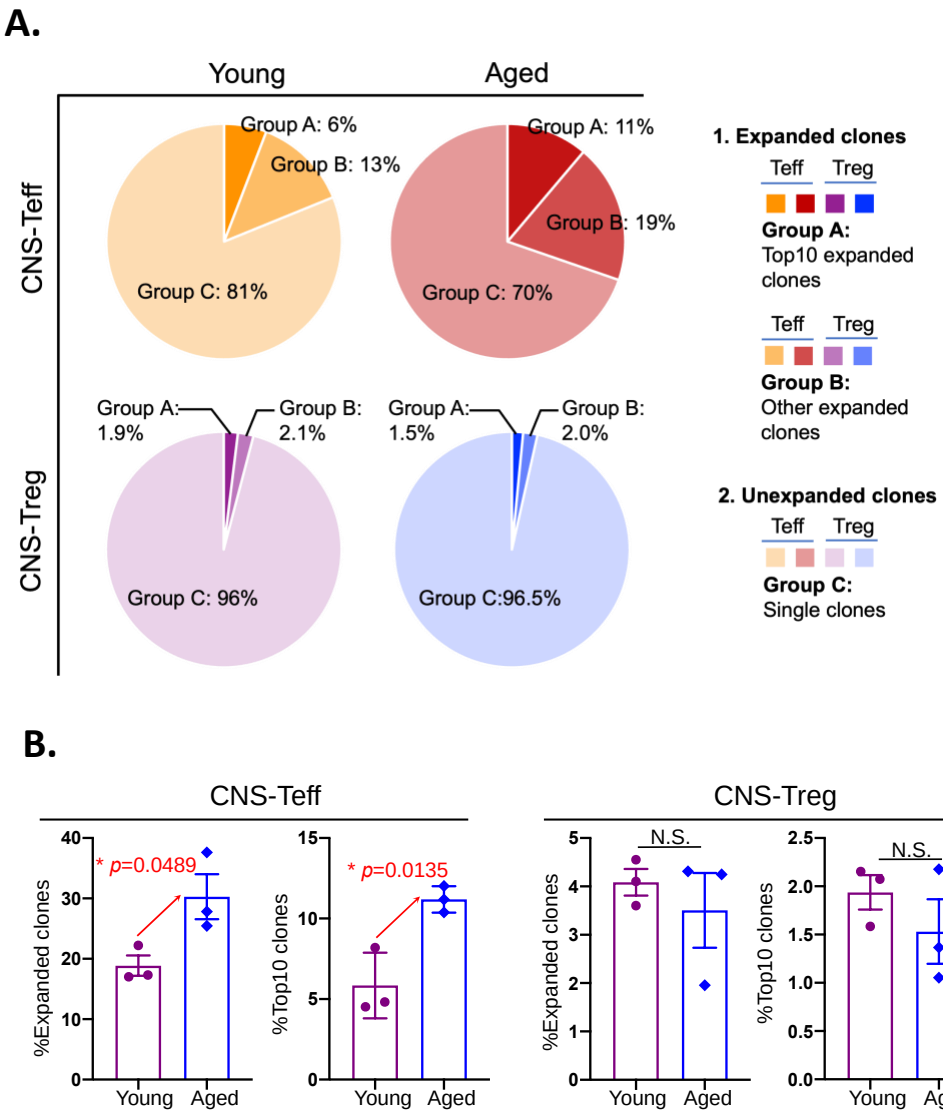


Figure 26. Clonal expansion assay in CNS-infiltrated CD4$^+$ T populations of young and aged EAE mice. Based on TCRαβ sequence similarity from the sc-RNA-Seq immune profile analysis, CNS-infiltrated Teff and Treg cells were divided into expanded T clones (more than one copy of each TCR sequence) and unexpanded T clones (unique TCR sequences, with clone size = 1). In the expanded T clones, we further divided them into Group-A: top 10 clones (10 most expanded TCR sequences) and Group-B: other expanded clones (all other TCR sequences with ⩾ 2 copies). **(A)** Pie charts show expanded clones (Groups-A and -B) and unexpanded clones (Group-C) of CNS-Teff (top two pies) and CNS-Treg (bottom two pies) cells in total CNS-CD4$^+$ T cells from young (left two pies) and aged (right two pies) EAE mice. **(B)** Summarized results of the frequencies of all expanded clones of CNS-Teff (left panel) and CNS-Treg (right panel) cells in total CNS CD4$^+$ T cells from young (cherry open bars) and aged (blue open bars) EAE mice. Each symbol represents one mouse. Data were analyzed by unpaired Student's *t*-test, and a statistically significant difference was considered to be $p < 0.05$.

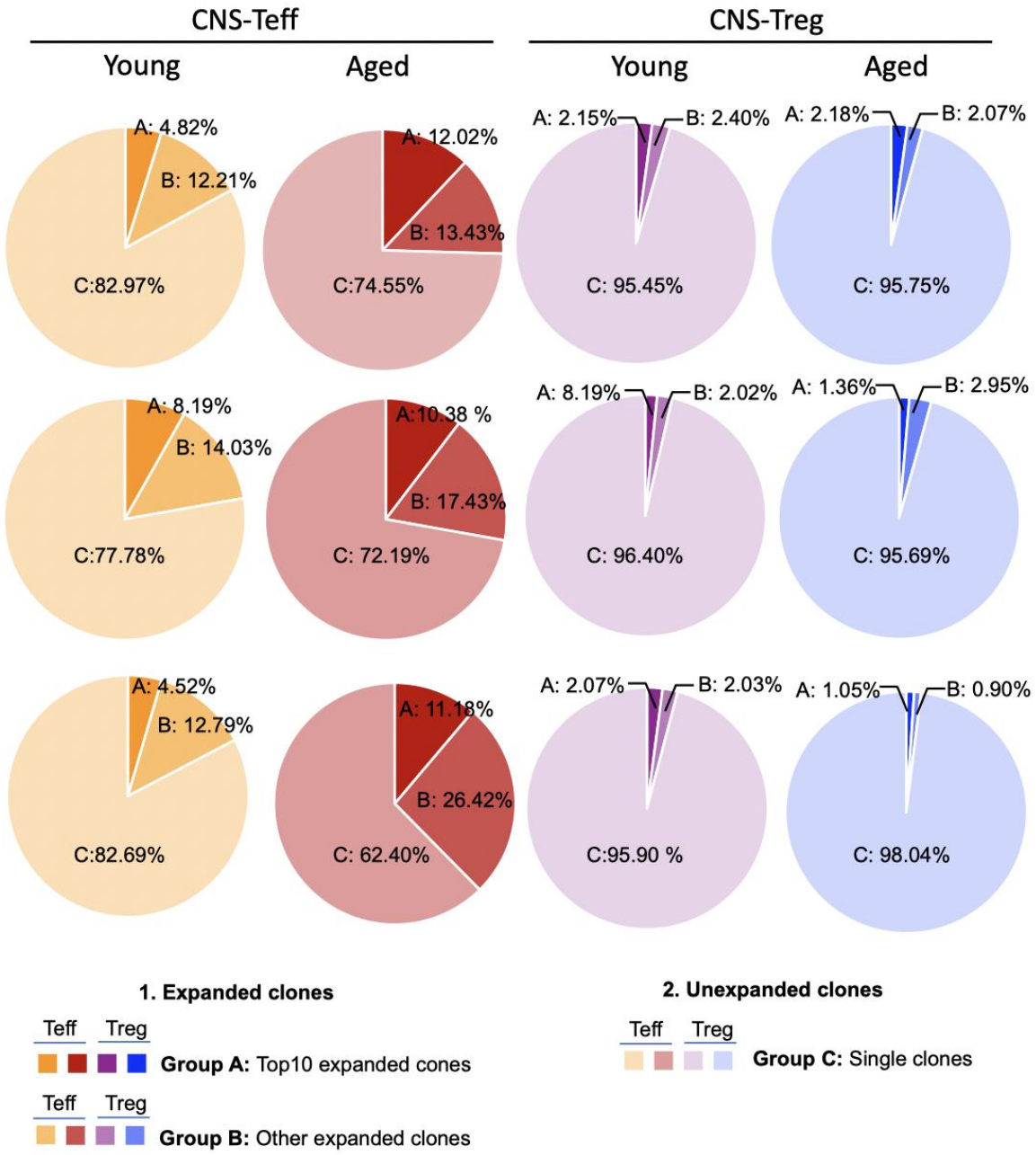


Figure 27. Clonal expansion in CNS-infiltrated CD4$^+$ T cells of three young and three aged EAE mice. Leftmost column: three individual pie charts of young CNS-Teff cell clonal expansion; second left column: three individual pie charts of aged Teff cell clonal expansion; second right column: three individual pie charts of young Treg cell clonal expansion; rightmost column: three individual pie charts of aged Treg cell clonal expansion. The results are for detailed elaboration of Fig. 26.

Chapter VI. Discussion, Limitation, and Future Direction

Typically, human MS disease develops in young (20 – 40 years old) females. However, late-onset MS whose manifestation occurs after the age of 60 years, has been reported in the elderly (127-130). The mean age of the MS population is rising (128), and it was reported that 14% of total MS patients were 65 years and older in 2010 (131). MS presents with at least four clinical sub-types. Two of them, progressive-relapsing MS (PRMS) and remission-relapsing MS (RRMS), have relatively mild symptoms, and are often seen in young patients (80% - 90%). However, the other two, primary progressive MS (PPMS) and secondary progressive MS (SPMS), have severe symptoms, and are often seen in aged (>65 years old) patients (~29% for PPMS and ~26% for SPMS), and young patients only account for 10% PPMS and rarely exhibit SPMS (128). Given that Treg cells are protective cells against autoimmunity (100, 101, 104), which combat IFN-γ-producing and IL-17-producing $CD4^+$ pathogenic cells and pathogenic $CD8^+$ T cells in the MS lesion, the severe symptoms in the aged patients are potentially attributed to Treg cell dysfunction, including altered distribution and increased plasticity.

Ample evidence shows that Treg cells play an ameliorative role in MS/EAE disease onset and severity (101, 226, 227), and transferring Treg cells into MOG-induced EAE mice attenuates disease (106). EAE severity is particularly correlated inversely with the frequency of MOG-specific Treg cells (107). However, accumulation of pTreg cells (107) with enhanced suppressive function (238) in aged individuals is accompanied by severe MS/EAE symptoms in aged humans (128) and mice (Fig. 9). This is not consistent with the role of Treg cells in suppressing uncontrolled immune reactions. Thus, this inconsistency encouraged us to investigate the underlying mechanism.

$CD4^+Foxp3^+$ Treg cells primarily act to suppress Teff cell-mediated aberrant antigen-specific and non-specific immune responses. However, accumulation of pTreg cells in the elderly is disadvantageous for anti-infection and anti-cancer immunity, and for certain neuroinflammation. This disadvantage indicates that excess Treg cells may be harmful. As mentioned earlier, in protection and recovery from CNS inflammatory disorder AD, one of the age-related neuroinflammatory diseases, excessive pTreg cells were shown to play a detrimental role (72). This is probably due to too many Treg cells accumulated outside the CNS (in the periphery), rather than inside the CNS (239), in addition to the existence of distinct Treg subsets (240, 241). Herein, using an aged EAE mouse model that resembles aged human MS disease, we found that aged EAE mice had an altered distribution of pan- and MOG-specific Treg cells inside and outside the CNS, compared to their young counterparts. Specifically, aged mice exhibited a high proportion of pan-pTreg and a low proportion of MOG-specific pTreg cells in their periphery, but low proportions of both pan- and MOG-specific CNS-Treg cells in the inflamed CNS (Fig. 13). Accumulation of pTreg cells outside the CNS and residing at the CNS-periphery boundaries, the BBB and CP, could impede immune cells, including Treg cells, trafficking into the inflamed CNS for recovery (72, 231).

As mentioned earlier, AD pathology could be alleviated by transient inhibition of Foxp3 expression in the accumulated peripheral $CD4^+Foxp3^+$ Treg cells in an aged AD mouse model (72). In our study, we transiently inhibited or depleted accumulated pTreg cells in aged EAE mice, which significantly ameliorated EAE disease and corrected Treg distribution (Figs. 18-21). The mechanism is likely that the accumulated pTreg cells suppress INF-γ producing cells residing at the CNS barrier CP, which directly block the gateway (72) for homeostatic leukocyte trafficking into the CNS (73, 228). IFN-γ is required for activation of the brain's CP for CNS immune

surveillance and repair (73, 231). For example, it is reported that immunization with a myelin-derived antigen activated the CP via inducing the CP to express IFN-γ and attract Th1 cells, thereby enhancing recruitment of immunoregulatory cells into the CNS to achieve attenuation of neuroinflammatory progression in a mouse model (242).

Treg cells are unstable and plastic, and this is especially observable during inflammatory autoimmune stimulation (200, 206). The unstable characteristics are exhibited by loss of Foxp3 expression in a proportion of mature Treg cells (243), and the plastic characteristics are exhibited by the production of pro-inflammatory cytokines IFN-γ or IL-17, along with Foxp3 expression. These altered Treg cells acquire an effector-like phenotype, and they can induce, rather than suppress, autoimmunity. These cells are commonly found in autoimmune-prone NOD mice and diabetic patients (203, 244), as well as in MS patients and EAE mice (245, 246). We found that in the aged, inflamed CNS, Treg cells exhibited higher plasticity than their young counterparts, observed as co-expression of INF-γ and/or IL-17 with Foxp3 (Fig. 24), which potentially results in reduced suppressive function and increased pathology (200, 235). The increased Treg plasticity in the aged EAE mice could be due to the synergistical effect of EAE induced acute inflammation and the pre-existing inflammaging, the latter of which is not possessed by young mice. Further, we observed differences in CNS-Teff clonal expansion, which reflects the reduced suppressive function of CNS-infiltrated Treg cells in aged EAE mice (Fig. 26, top pies). The clonal expansion of CNS-infiltrated Treg cells was almost the same between young and aged EAE mice, whereas, the clonal expansion of CNS-Teff cells was significantly increased in the aged EAE mice. The lower Treg clonal expansion reflects their biological feature. Our data also showed that expanded CNS-infiltrated Treg clones (clone size ≥ 2) is only accounting for 3~4% of total CNS-infiltrated Treg clones in both young and aged mice (Fig. 26A, bottom pie charts). The higher clonal

expansion of CNS-Teff cells in aged, inflamed CNS could explain the lower suppressive function of the CNS-Treg cells, since this reflects a disruption of the normal Treg/Teff balance. In addition to self-antigen-driven inflammatory autoimmune stimulation (200, 206), the age-related, chronic, systemic inflammation (inflammaging) may play a synergistic role to enhance CNS-Treg plasticity in aged EAE mice compared to young counterparts. However, this synergistic role is our future work, and it needs further investigation. One technical limitation is that we were not able to identify the clonal expansion of MOG-specific T cells among the whole T cell population using sc-RNA-Seq approach, since unique sequence-based MHC-II MOG-Dextramer reagent is under development.

Although $CD4^+$ T cells, both $CD4^+$ Teff and $CD4^+Foxp3^+$ Treg cells, are the traditional primary actors in MS/EAE pathogenesis and immunoregulation, emerging evidence shows that $CD8^+$ T cells also contribute to immunopathology (by cytotoxic $CD8^+$ T cells) and immunoregulation (by $CD8^+$ Treg cells), and can either exacerbate or mitigate brain inflammation during CNS autoimmunity (111, 112). Regarding MS/EAE immunopathogenesis, myelin-specific $CD8^+$ T cells exacerbate brain, though not spinal cord, inflammation via a Fas ligand-dependent mechanism to promote lesion formation in the brain (116). In addition, IL-17A secreting $CD8^+$ T cells (termed Tc17 cells) in the CNS support Th17 cell-mediated autoimmune encephalomyelitis (114, 236). In aged neurogenic niches that comprise neural stem cells, $CD8^+$ T cells are increased and inhibit the proliferation of neural stem cells (113). These pathogenic $CD8^+$ T cells are detrimental for the recovery of demyelinating lesions in aged MS/EAE disease. Our results show that $Tc17^+$ cells expressed increased levels of proinflammatory cytokine genes in the aged EAE CNS (Fig. 24), which was consistent with severe symptoms and pathology in aged EAE mice. However, regarding the immunoregulation of $CD8^+$ Treg cells in MS/EAE, although the concept

of $CD8^+$ Treg cells is not unanimously accepted, the function of $CD8^+$ Treg cells in MS/EAE has received attention (115, 120, 121). The main focus of studies of $CD8^+$ Treg cells in MS/EAE is based one young patients or young animals, and there are many unanswered questions regarding how these cells contribute to CNS autoimmune inflammation in the elderly. Therefore, studies in the role of $CD8^+$ Treg cells in the elderly during late-onset MS/EAE disease are our future work and need to be investigated.

In summary, the results of our project provide insights into how accumulated aged polyclonal $CD4^+Foxp3^+$ pTreg cells in an inflammatory condition do not ameliorate, but are detrimental for CNS repair processes in autoimmune neuronal inflammation of aged MS, which is demonstrated in the animal model EAE.

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