Murine autoimmune hearing loss mediated by \(CD4^+\) T cells specific for \(\beta\)-tubulin

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Abstract
Autoimmune inner ear disease is described as progressive, bilateral although asymmetric, sensorineural hearing loss and can be improved by immunosuppressive therapy. We showed that the inner ear autoantigen \(\beta\)-tubulin is capable of inducing experimental autoimmune hearing loss (EAHL) in mice. Immunization of BALB/c mice with \(\beta\)-tubulin resulted in hair cell loss and hearing loss, effects that were not seen in animals immunized with control peptide. Moreover, the EAHL model showed that \(\beta\)-tubulin responsiveness involved \(CD4^+\) T cells producing IFN-\(\gamma\), and T cell mediation of EAHL was determined by significantly increased auditory brainstem response after adoptive transfer of \(\beta\)-tubulin-activated \(CD4^+\) T cells into naive BALB/c recipients. The potential mechanisms responsible for the observed pathology of EAHL can be attributed to decreased frequency and impaired suppressive function of regulatory T cells. Our study suggests that EAHL may be a T cell-mediated organ-specific autoimmune disorder of the inner ear.

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Introduction

Autoimmune diseases are characterized by a deleterious reaction of the immune system to autoantigens and self-tissue and lead to chronic inflammation and subsequent damage of the affected tissues. Little is known about the reasons underlying the immune system's loss of self-tolerance [1] and the causes for the tissue preference of certain disease entities. While most autoimmune diseases are systemic disorders that can affect the whole body, they usually show a preference for certain tissues or organ systems.

Some autoimmune conditions appear to be even more selective by affecting primarily very specific and even immune-privileged tissues, such as the cochlea in autoimmune diseases of the inner ear, as was first described by McCabe in 1979 [2]. He described patients with progressive, bilateral, synchronous or sequential, sensorineural hearing loss who responded to immunosuppressive therapy and who also tested positive for laboratory tests, clinical manifestations, and immunological tests prescribed for autoimmune hearing loss [3].

Abbreviations: ABR, auditory brainstem responses; AIED, Autoimmune inner ear disease; AISHL, autoimmune sensorineural hearing loss; DPOAE, distortion product of oto-acoustic emission; EAHL, experimental autoimmune hearing loss; ICA, incomplete Freund's adjuvant; iTreg, inducible regulatory T cells; LN, lymph node; MD, Ménière's disease; nTreg, naturally occurring regulatory T cells; OVA, ovalbumin; SPL, sound pressure level; Treg, regulatory T cells.

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Autoimmune inner ear disease (AIED) can occur as a separate entity or as part of an ear disease, such as Ménière’s disease (MD), otosclerosis, or vestibular neuritis. It can also be seen in association with other autoimmune diseases such as systemic lupus erythematosus, disseminated vasculitis, Sjögren’s syndrome, systemic sclerosis, myasthenia gravis, Hashimoto’s thyroiditis, Goodpasture’s syndrome, strial atrophy, and rheumatoid arthritis, indicating that AIED may present as a localized primary disease or be present in association with a systemic autoimmune disorder, being referred to as secondary. Approximately 30% of patients with AIED have a systemic autoimmune disease [4].

However, the pathological mechanism(s) of AIED is still unknown. An autoimmune reaction to the inner ear structures could result from one of several possible pathogenic factors. Many autoantigens have been implicated as possible causal antigens in AIED: heat shock protein 70 [5,6], collagen II [7,8], cochlin [9,10], and, most recently, β- tubulin [11–15].

Autoantibodies have long been suspected of mediating AIED. Sera from AIED patients frequently contain Ab capable of immunostaining human temporal bone sections of the inner ear [16,17]; however, the antigen specificity is still unclear. Autoreactive T cells have also been implicated in AIED as they have been shown to elicit increased inhibition of leukocyte migration [18], increased proliferation [19], and frequency of IFN-γ secretion in response to the human inner ear homogenate [20]. In animal studies, cochlear pathology and/or hearing loss were observed in guinea pigs after immunization with the inner ear homogenate [21–23], and autoimmune disease of the inner ear developed in Lewis rats adoptively transferred with T cell lines specific for the inner ear homogenate [24,25].

The objective of this study was to investigate the immune reactivity to the inner ear components in mice with EAHL. We selected β- tubulin as a candidate autoimmune target protein, because Yoo et al. demonstrated that 67 (59%) of 113 patients with MD had antibodies to a 55-kDa protein β- tubulin in guinea pig inner ear extract [11–15]. Moreover, immunohistological studies showed that β- tubulin appeared to be the highly expressed protein in the inner ear tissues such as hair cells, supporting cells, spiral ligament of stria vascularis, neural pathway of the cochlea, as well as the spiral ganglion, indicating that β- tubulin is a fundamental protein in guinea pig inner ear [11,14]. Nevertheless, inner ear immunization with β- tubulin changed β- tubulin spatial distribution in specific structures [14] and caused degeneration of the spiral ganglion [14], thereby affecting the functions of microtubules in the stria vascularis and the spiral ganglion. More recently, Cai et al. developed a form of EAHL by immunizing BALB/c mice with recombinant β- tubulin [15]. These mice developed substantial hearing loss and loss of hair cells in the basal turn of the cochlea. However, peripheral tolerance could be induced by oral administration of low-dose β- tubulin antigen (Ag) in an animal model of AIED [15]. This treatment showed less hearing loss and less inner ear damage, decreased IFN-γ secretion in response to β- tubulin Ag, and demonstrated an effective, Ag-specific method to suppress EAHL.

Tubulin is a major constituent protein of microtubules, which are prominent structures in the sensory and supporting cells of the inner ear. Microtubules play an active role in structural support, intracellular transport, and cell motility and mitosis [26]. The possible functions of microtubules in hair cells include morphogenesis and maintenance of cell shape, intracellular transport, providing a possible substrate for motility, and mechanical support of structures associated with sensory transduction [27]. Individual microtubules are composed of heterodimers of α- and β- tubulin subunits [28]. β- tubulin was found in the cochlea of the guinea pig [11] and gerbil [29], as well as in the avian inner ear [30]. However, the pathological changes induced by β- tubulin immunization are still unknown.

In the present study we confirmed that β- tubulin is capable of mediating EAHL following the immunization of BALB/c mice and causing lesions in the cochlear hair cells and cochlear damage of the spiral ganglion. We also show that EAHL could be mediated by the passive transfer CD4+ T cells specific for β- tubulin to naive mice. Moreover, we also demonstrate that the decreased frequency and impaired suppressive function of regulatory T cells (Treg cells) are associated with the pathogenesis of β- tubulin mediated EAHL.

Materials and methods

Mice and immunization

Female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used in this study, and auditory brainstem response (ABR) were measured bilaterally, both preexperiment and posttreatment, for all the mice to ensure their normal hearing function. Mice were maintained in the animal facility at the University of Tennessee Health Science Center, according to the institutional guidelines for animal care and use. These studies were approved by the Institutional Animal Care and Use Committee of the University of Tennessee. At 6 weeks of age, mice were immunized subcutaneously with 300 μg of β- tubulin (recombinant full-length human β- tubulin, Abcam, Cambridge, MA) or 300 μg of OVA (Sigma-Aldrich, St. Louis, MO) emulsified with an equal volume of complete Freund’s adjuvant (CFA, Difco Laboratories, Detroit, MI) containing 2 mg/ml of H37Ra Mycobacterium tuberculosis (Difco). The mice were given boosters by subcutaneous injection with β- tubulin or OVA emulsified with incomplete Freund’s adjuvant (ICA, Difco) twice at 1-week intervals, 2 weeks after the initial immunization.

Hearing tests

During ABR measurements, mice were anesthetized with Avertin (500 mg/kg bodyweight). Far field auditory brainstem evoked response was conducted in a sound-attenuating booth. ABRs were recorded between subcutaneous needle electrodes in vertex as active, posterior bulla as reference, and the ground electrode placed at the lower back. Click and tone burst stimuli of 8, 16, and 32 kHz were generated and delivered to both ears through a high-frequency transducer. A maximum sound pressure level (SPL) was stimulated in tone bursts of 100 dB. The evoked potentials were amplified 5000 times and averaged from 600 evoked responses for the first 10-ms period following stimulation. Auditory thresholds were determined by increasing the sound intensity of the tone burst for each frequency stimulus and verified twice. Auditory evoked potential amplitude was calculated from all traces
between the maximum intensity of 100 dB and the minimum intensity as the hearing threshold was determined.

**In vitro cytokine production and lymphocytes proliferation**

Six weeks after β-tubulin immunization, inguinal and axillary lymph node cells (LN cells) were harvested. Cells (2 × 10^6 cells/well) were cultured in 96-well flat-bottomed plates (Costar, Corning, NY) in RPMI 1640 medium supplemented with 5% FCS (Gibco, Paisley, UK), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 10 U penicillin/streptomycin (Gibco), and stimulated with β-tubulin in serial 10-fold dilutions. Positive control wells contained 2 μg/ml anti-mouse CD3 (BD Biosciences), and negative control wells contained only PBS. Supernatants were harvested after 48 h and stored at −70 °C for cytokine measurement. Cells were pulsed with [³H]-thymidine during the last 18 h of the 72-h assay, harvested, and counted for [³H]-thymidine incorporation (Packard Instrument, Boston, MA). Stored supernatants were analyzed for IL-4, IL-10, IFN-γ, and TGF-β by ELISA, per the manufacturer’s protocol (R&D Systems).

**Flow cytometry**

Six weeks after immunization, LN cells were mechanically teased into single-cell suspensions and reactivated in vitro with 100 μg/ml β-tubulin for 3 days. Cultured cells were washed thoroughly and centrifuged on density-gradient medium Lympholyte-M (Accurate Chemical and Scientific Corp., Westbury, NY) to collect cells from the interface. After multiple washes, cells were incubated at 4 °C with Abs specific for mouse CD4 or isotype control Ab (BD Biosciences). The CD4^+CD25^-Foxp3^-expressing T cells were identified by staining LN cells with PE-labeled anti-CD4 (RM4-5, eBioscience, San Diego, CA) and APC-labeled anti-CD25 (PC61.5, eBioscience). For intracellular staining of Foxp3, cells were fixed and permeabilized according to the manufacturer’s instructions before incubation with FITC-labeled anti-mouse Foxp3 (FJK-16s, eBioscience). Appropriate isotype-matched control antibodies were used to determine nonspecific staining. Labeled cells were washed with PBS, and at least 10,000 cells were analyzed from each sample by flow cytometry with an LSR II (BD Biosciences). The percentage of Treg cells was determined after gating on CD4^- T cells and using FlowJo software (Tree Star, Ashland, OR).

**In vitro suppression assay**

Isolation of mouse CD4^+, CD4^+CD25^-, and CD4^+CD25^- T cells was performed by using a mouse Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach) according to the manufacturer’s instructions. Briefly, CD4^- T cells were first enriched by negative selection (depleting CD8a, CD11b, CD45R, CD49b, and Ter-119-positive cells) with MACS. The CD4^- T cells were incubated with magnetic beads conjugated with an anti-CD25 mAb to separate CD4^+CD25^- and CD4^+CD25^- T-cell subpopulations. The purity of the resulting T-cell subpopulations was higher than 95% by flow cytometry. Proliferation assays were performed in triplicate by culturing CD4^+CD25^- cells (responder, 5 × 10^4 from the LN cells of β-tubulin immunized mice), CD4^+CD25^- T cells (suppressor, 5 × 10^4 from the LN cells of β-tubulin immunized mice, OVA control or naive mice) in 96-well plates with irradiated APCs (5 × 10^4 from splenocytes of normal BALB/c mice) for 72 h at 37° in complete medium. Cultures were stimulated by β-tubulin (100 μg/ml). After 3 days, 1 μCi/well [³H]-thymidine was added. Incorporation of [³H]-thymidine was assessed after 16 h of additional culturing.

**Histology**

After anesthesia with Avertin, animals were perfused through the heart with a solution of warm 0.1 M phosphate buffer, followed by a warm buffered 1.5% paraformaldehyde solution for 2 min. This was followed by a second fixative containing cold 4% paraformaldehyde for 25 min. The animals were decapitated, the brain and brainstem were exposed, and the entire head with temporal bone was immersed in 10% neutral formaldehyde solution and stored overnight. Temporal bones were dissected out and postfixed in a fresh fixative at 4 °C overnight. After rinsing with PBS, the tissues were decalcified in Tris buffer (pH 7.2) containing 10% EDTA at 4 °C for a week. Then the temporal bones were dehydrated and embedded in paraffin. Groups of 12-μm thick sections were collected using a microtome and mounted in serial order. All sections were mounted and stained with standard H&E and examined for evidence of defect or degeneration. All sections were examined with a light microscope (Carl Zeiss Axioskop 2 plus HAL 100 with final enlargement ×400). Digital images of sections were obtained using a camera (Leica) linked to a Windows computer.

**Statistical analysis**

Data were analyzed using ANOVA or Student’s t-test to compare differences between the OVA and experimental groups.

**Results**

**Hearing loss following active immunization with β-tubulin**

To determine whether β-tubulin exposure was associated with hearing loss, female BALB/c mice were immunized with β-tubulin or OVA (Figure 1), and ABRs were performed 2 and 6 weeks after the final booster. Figures 2A and B show no differences in the ABR thresholds (P=0.08 and P=0.15) between the OVA-immunized control mice and naive mice (untreated mice). Thus, the OVA-immunized control mice showed no spontaneous hearing loss and are provided in each figure as a reference. Figure 2A shows a significant increase in the ABR click and pure tone thresholds in mice immunized with β-tubulin 2 weeks after the final booster at all frequencies tested from 8 kHz to 32 kHz in comparison with the OVA-immunized controls (P=0.002). Figure 2B shows a significant increase in the ABR click and pure tone thresholds in mice immunized with β-tubulin 6 weeks after the final booster at all frequencies tested from 8 kHz to 32 kHz in comparison with the OVA-immunized controls (P=0.003). Furthermore, similar results have been observed by the distortion product of oto-acoustic emission (DPOAE) measurement (data not shown).
Increased frequencies of β-tubulin-responsive T cells in EAHL model and hearing loss in naive mice following adoptive transfer of activated T cells specific for β-tubulin

Because CD4⁺ T cells are believed to play a central role in initiating and perpetuating autoimmune diseases, we first identified the important role of CD4⁺ T cells in response to β-tubulin in EAHL. 6 weeks after β-tubulin immunization, LN cells were harvested and stimulated in vitro with β-tubulin to study the nature of their recall response to Ag challenge.

Figure 3A shows no differences between the OVA control mice and naive mice in recall proliferative responses to β-tubulin (P=0.35). Thus, the OVA controls are provided as a reference in each figure. Furthermore, lymphoproliferative responses of LN cells from the EAHL mice were observed when the cells were exposed to progressively increasing doses of the β-tubulin preparation, with a dose effect (Figure 3A). In addition, we found that β-tubulin was able to elicit substantial recall proliferative responses from β-tubulin-primed LN cells (P<0.021), whereas the OVA-immunized lymphocytes were relatively nonimmunogenic. The recall proliferative response of T cells in response to 100 μg β-tubulin was enhanced 6-fold in mice that were immunized with β-tubulin in comparison with the OVA control mice (Figure 3A). Flow-cytometry analysis of β-tubulin-primed LN cells, which were stimulated in vitro with β-tubulin, showed that β-tubulin (Figure 3B) preferentially activated CD4⁺ T cells.

We next determined whether purified CD4⁺ T cells, specifically activated with β-tubulin in vitro, were capable of passively transferring EAHL into irradiated naive BALB/c female mice. 2 weeks after immunization with either β-tubulin or OVA, splenocytes were activated in vitro with 100 μg/ml of β-tubulin. After 3 days, CD4⁺ T cells were purified greater than 95% from activated cultures by magnetic bead separation and injected i.v. into γ-irradiated naive recipients at 2×10⁷ cells/mouse. 6 weeks after transfer, the ABR thresholds were significantly increased at all frequencies tested in the ABR click and pure tone thresholds in mice transferred with CD4⁺ T cells specific for β-tubulin when compared with naive controls (P=0.0069) and mice transferred with CD4⁺ T cells specific for OVA (P=0.0017, Figure 3C). Naive mice and mice transferred with CD4⁺ T cells specific for OVA showed no differences in the ABR thresholds (P=0.15). This shows that purified CD4⁺ T cells specific for β-tubulin are capable of mediating EAHL in mice.

β-tubulin induces deviation of Th1 and Th2 cytokine responses

In general, Th1-type cytokines such as IFN-γ perpetuate the inflammatory response in the lesions of autoimmunity, whereas Th2-type cytokines such as IL-4 represent one way in which the inflammatory response may be controlled. ELISA analysis of culture supernatants showed that recall responses to β-tubulin (Figure 4) involved the proinflammatory Th1-like phenotype, compared to the OVA control mice. β-tubulin elevated the production of IFN-γ in week 6 after immunization (Figure 4A). There were no differences for β-tubulin-reactive...
Th2-like cytokines such as IL-4 between the OVA control mice and mice treated with β-tubulin (Figure 4B). Moreover, β-tubulin-immunized mice showed downregulated levels of β-tubulin-reactive IL-10 (Figure 4C) and TGF-β (Figure 4D) as compared with the OVA control mice, indicating that β-tubulin-induced EAHL decreased the production of IL-10/TGF-β-producing T cells.

Decreased frequency and impaired suppressive function of Treg cells in the EAHL model

Several studies have indicated that Treg cells confer significant protection in controlling autoimmunity by suppressing self-reactive T cells (32–34). Therefore, defects in Treg development, maintenance, or function have been associated with autoimmune diseases. The observed upregulation of the Th1 responses, together with the decreased levels of regulatory cytokine IL-10 and TGF-β, prompted us to hypothesize that the dysfunction of Treg cells may be responsible for the observed pathology of EAHL. Therefore, we compared the proportion and suppressive function of Treg cells among normal, β-tubulin-treated, and the OVA control groups, in view of the critical role of the Treg cells in restraining autoaggressive T cells in experimental settings.

In our studies, the EAHL mice significantly decreased the frequency of CD4+CD25+ T cells; the EAHL mice had significantly lower number (5.5± 0.9% of total FACS-sorted CD4+ T cells) of CD4+CD25+ T cells compared with 10.4 ± 1.5% (of total FACS-sorted CD4+ T cells) of CD4+CD25+ T cells from the OVA control mice and 10.9±2.5% (of total FACS-sorted CD4+ T cells) of CD4+CD25+ T cells from naive mice (P=0.01 and P=0.008, respectively).

Constitutive expression of the transcriptional repressor Foxp3 has been considered to be a major phenotypic marker of the Treg cells. Its expression was detected among the normal, β-tubulin-treated, and OVA control mice. The CD4+ lymphocyte population from the EAHL mice had a significantly lower number of cells with Foxp3+ expression (6.7±1.7% by FACS) compared to the naive mice and the OVA control mice (14.5±5.1% and 15.6±6.3% by FACS, respectively, P=0.012 and P=0.01).

Moreover, we found that EAHL evidently decreased the proportion of CD4+CD25+ Foxp3+ cells in β-tubulin-immunized mice (Figure 3A). The mean ±SE percentage of CD4+CD25+ Foxp3+ cells among the CD4+CD25+ Foxp3+ cells from naive mice was (4.2±0.5%) and (4.0±0.5%) in EAHL, nearly a 2-fold decrease over that in the OVA control mice and naive mice (P=0.005 and P=0.001). There was no statistically significant difference in the frequency of CD4+CD25+ Foxp3+ cells between the OVA and naive groups (P=0.25).

The regulatory properties of CD4+CD25+ T cells were evaluated by testing their ability to suppress the proliferative responses of CD4+CD25− T cells. CD4+CD25− T cells from β-tubulin-treated mice were cocultured with CD4+CD25+ T cells from the EAHL mice, OVA control mice or naive mice with β-tubulin (100 μg/ml). Figure 3B shows that CD4+CD25+ Treg cells from the EAHL mice failed to suppress the proliferation of autologous CD4+CD25+ effector T cells, whereas CD4+CD25+ Treg cells from the OVA controls and naive mice could suppress the proliferative response of CD4+CD25+ effectors from the
EAHL mice. These data indicate that decreased frequency and impaired suppressive function of Treg cells are associated with the pathogenesis of EAHL.

Hair cell loss in β-tubulin-immunized mice

Six weeks after β-tubulin immunization, cochlea were harvested and stained with H&E for histological analysis (Figure 6). Cochlear cross-sections from naive mice (Figures 6A and D) and the OVA control mice (Figure 6B and E) revealed a normal density of spiral ganglion cells (Figures 6A and B), as well as three outer hair-cell rows with one row of inner hair cells (Figures 6D and E) in the basal turn of the cochlea. Cross-sections from the β-tubulin experimental group revealed a drastic and sizable degeneration in the spiral ganglion cell population of the organ of Corti (Figure 6C). Whole mount preparations of the cochleae showed that significant hair cell loss had occurred in β-tubulin-immunized mice (Figure 6F). The ABR measurements revealed severe hearing impairment in β-tubulin-immunized mice and could explain the observed hearing phenotype. No hair cell loss in the OVA control mice was found; thus, the OVA control mice had normal hearing compared with the β-tubulin-immunized mice.

Discussion

In the current study, we showed that the inner ear antigen β-tubulin is capable of inducing EAHL in mice by causing damage to the organ of Corti and inducing hearing loss. Moreover, EAHL developed in naive mice adoptively transferred with β-tubulin-specific CD4+ T cells, suggesting that Ag-primed CD4+ T cells appears to be critical for the development of EAHL. This view does not preclude a role for autoantibodies in EAHL, but the low 1/80–1/160 serum antibody titer generated in response to immunization with β-tubulin however appears to be inadequate to transfer disease. It is possible that autoantibodies may contribute to- or exacerbate preexisting inner ear pathology in this model.

The Th1 cytokines are recognized as proinflammatory and represent the driving force in the T cell-induced autoimmune process. In this study, we showed that the cytokine profile from β-tubulin-immunized animals was clearly changed when compared to the OVA-immunized control animals. The integrated results indicate IFN-γ enhancement and no production of IL-4. Furthermore, the cytokine profiles recorded in the LN cells also showed that β-tubulin-immunized animals had reduced production of TGF-β and IL-10.

However, although our studies and other laboratories’ publications suggested that the development of EAHL is related to exposure to the inner ear antigens, they did not explain how tolerance to these self-antigens was broken. Although the relationship between impaired tolerance and progression of autoimmune disease is unclear, it is likely that deregulation of the immune system is involved. Autoreactive T cells are eliminated primarily in the thymus by negative selection (central tolerance), but some may escape from negative selection to be released into the periphery. These self-reactive T cells are exquisitely regulated, and their activation can result in autoimmune diseases. Xia et al. [31] reported the defective regulatory function of CD4+CD25+ T cells in patients with autoimmune sensorineural hearing loss (AISHL), indicating its involvement in the pathogenesis of AISHL.

Recently, accumulating evidence suggests that Treg cells play a critical role in maintaining peripheral homeostasis and establishing controlled immune responses [32–34]. There are two classes of Treg cells within CD4+ T cells: (1) the naturally occurring, thymus-selected CD4+CD25+ FoxP3+ Treg cells
(nTreg) [35], which develop in the thymus and are present in normal naive mice and healthy individuals from birth, and (2) the inducible Treg cells (iTreg) including Tr1 and Th3 cells, which are generated in the periphery under various tolerogenic conditions [36]. nTreg cells constitutively express both CD25 [37,38] and Foxp3 [39], and Foxp3 mutations result in the loss of Treg cells and underlie a homologous autoimmune lymphoproliferative disorder [40–42].

In our studies, β-tubulin-immunization in animals decreased the expression of Foxp3 and altered the ratio of CD4+CD25+ cells to CD4+CD25− cells. The number of nTreg cells decreased in EAHL, possibly as a result of altered transcriptional regulation: decreased Foxp3 could explain low Treg frequency. A Treg defect may help explain the increased autoreactive T cells and the development of EAHL. The most widely accepted marker for nTreg cells is Foxp3. The precise function of Foxp3 is not known, but an absence of Foxp3 in humans results in IPEX syndrome [43,44]. Foxp3 is crucial for the suppressive capacity of the Treg activity in allergic asthma and autoimmune diseases. In patients with multiple sclerosis, CD4+CD25+ Treg cells have reduced levels of Foxp3 expression, which increased after treatment with a copolymer-1 [45]. Furthermore, this study indicated that the suppressive capacity of Treg cells from EAHL model was impaired. Also, β-tubulin-immunized animals had reduced the production of immunoregulatory cytokine IL-10 and TGF-β, indicating that β-tubulin-immunization decreases the number of IL-10-producing T cells (Tr1-like cells) [46] and TGF-β-producing T cells (Th3-like cells) [47]. These results add to the possibility that Treg cells are dysfunctional in the EAHL mice. Loss of T-Cell regulatory function appears to be an important factor in the pathogenesis of EAHL.

In our experimental setting, no signs of additional inflammatory disorders have been found. In terms of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, we did not detect any EAE disease during the three month period over which the β-tubulin immunized mice were observed. Moreover, we did not observe any changes in body weight, color, and movement in β-tubulin-immunized mice.

In summary, our studies indicated that β-tubulin auto-reactivity is significantly enhanced in mice with EAHL. This β-tubulin responsiveness involves β-tubulin-specific CD4+

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**Figure 5** Decreased frequency and impaired suppressive function of Treg cells in the EAHL model. Six weeks after β-tubulin immunization, LN cells were harvested. (A) Phenotypical alteration of CD4+CD25+ Treg cells from the EAHL mice. To evaluate the proportion of Treg cells in the experimental groups, live lymphocytes were gated from the LN cells of experimental groups. The cell surfaces were stained with CD4-PE and CD25-APC Ab. After permeabilization buffer treatment, the cells were stained with anti-mouse Foxp3-FITC Ab. The data depict the means±SE from five mice/group. (B) CD4+CD25+ T cells from the EAHL mice failed to suppress proliferation of CD4+CD25− T cells. CD4+CD25+ T cells purified from the LN cells of the EAHL mice (E), OVA control mice (O), or naive mice (N) by magnetic antibody cell sorting. CD4+CD25− T cells isolated from β-tubulin immunized mice as responders. CD4+CD25+ T cells alone, CD4+CD25− T cells alone, and various CD4+CD25+ T cells cocultured with CD4+CD25− T cells in the presence of autologous irradiated APCs and β-tubulin (100 μg/ml). After 3 days, 1 μCl/well [3H]-thymidine was added. Incorporation of [3H]-thymidine was assessed after 16 h of additional culturing. Values are the means±SE of five mice per group. P=0.005 versus EAHL mice.
T cells, as well as decreased frequency and impaired suppressive function of Treg cells in the etiopathogenesis of β-tubulin-induced EAHL. Thus, providing a useful mouse model for clarifying our understanding of AIED, and defective regulatory function of Treg cells in the EAHL model may serve as a platform for developing contemporary immunomodulatory adjunct therapies for AIED.

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